THE EFFECTS OF ADRENAL INSUFFICIENCY AND OVERDOSAGE WITH DOCA ON BILATERALLY ADRENALECTOMIZED SHEEP[1]

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There have been extensive investigations on the effect of adrenalectomy in man, dog, cat and rat, but no comparable metabolic studies have been made on the sheep.

In an endeavour to interpret the mechanism of the remarkable parotid salivary adaptation to sodium depletion which occurs in the sheep (Denton, 1956, 1957), it became necessary to establish the effect of adrenalectomy and of the administration of adrenal hormone on the electrolyte metabolism of normal sheep and on those with a permanent unilateral parotid fistula. It was found that adrenal insufficiency in the sheep involved essentially similar electrolyte derangement as in other animals. This was so whether or not the animal had a parotid fistula. The existence of the parotid fistula, however, by allowing measurement of salivary $Na^+ : K^+$ ratio, gave additional information on the change in electrolyte metabolism in adrenal insufficiency, and during variation of desoxycorticosterone acetate (DOCA) dosage.

A notable feature of hormone withdrawal in the adrenalectomized sheep was the profuse diuresis which occurred during the first two days.

METHODS.

Crossbred Merino sheep (ewes and wethers) were used. The final sequence of surgical preparation of the animals was as follows: A right adrenalectomy was performed. Several weeks later a unilateral parotid fistula (Denton, 1957) was made, and in four animals a carotid loop (van Leersum, 1911) was made on the same side. One to two months later a left adrenalectomy was performed. The individual operation histories will be described below.

Pre-operative preparation. Two days before operation the animal was given half its usual food ration, and on the day before operation no food or water was given. Before the left adrenalectomy, the following additional measures were adopted. For two days prior to

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operation and on the morning of operation, cortisone (50-100 mg.) and DOCA (5-10 mg.) were administered by intramuscular injection. The usual $NaHCO_3$ supplement was given by rumen tube to the sheep with fistulae.

Anaesthesia (Denton, 1957).

Adrenalectomy. The animal was placed on its side with the hind legs drawn down as far as possible towards the foot of the operating table. A sandbag was placed transversely under the animal at the level of the costal margin. The skin incision was made approximately 15 cm. long from the tip of the spinous process parallel to and about 2 cm. below the costal margin. The abdominal and vertebral muscles were divided in the same line. The adrenal gland was then exposed extraperitoneally by sweeping the retroperitoneal tissues forward. The insertion of a Devine operating frame (Devine, 1940) gave very good exposure of the operation field.

The vessels entering the cortex of the adrenal gland were divided between double ligatures and the sympathetic nerves were cut. This left the gland attached only by the main hilar vein to the inferior vena cava on the right side, or to the left renal vein on the left side. Only once was it possible to divide the hilar vein between ligatures, as it is normally wide and extremely short. Usually the gland was removed by applying a special clamp along the length of the main vessel close to its edge and cutting the hilar vein close to the gland with a scalpel. The resultant 1-2 cm. slit in the main vessel was then closed by continuous lateral suture using atraumatic catgut or silk. The abdominal wall was closed in layers.

Post-operative care. The sheep recovered rapidly from the operation. Often after one hour they were standing and feeding. Crystalline penicillin (1,000,000 U.), procaine penicillin (500,000 U.) and streptomycin (0·5 gm.) were given twice daily for the following three days. If antibiotics were continued for longer periods the sheep developed diarrhoea, presumably because of alteration of ruminal micro-organisms. In all instances the wounds healed uneventfully.

For details of post-operative hormone management see "Results" section.

Procedure of observation of animals in a metabolic cage has been described in detail elsewhere (Denton, 1956). Briefly, each morning at the same time the 24-hour specimen of saliva and urine was collected. If the animal had a fistula, the daily supplement of $NaHCO_3$ was given into the rumen, and then the animal was weighed and a blood sample taken. Immediately afterwards the daily dose of DOCA and cortisone was given, and the animal was fed. It follows, therefore, that the compositions of plasma recorded were those prevailing 24 hours after the injection of hormone (Table 1). Control periods were considered to end 24 hours after the previous dosage of hormone.

Diet. Sheep Adr. 1 was fed on a daily ration of oaten chaff (0·4 kg.) and lucerne chaff (0·4 kg.). The electrolyte composition was—oaten chaff (mean of six aliquots of the food) $Na^+ = 167$ (range 160-170), $K^+ = 199$ (195-205), $Cl^- = 206$ (195-215) m.equiv./kg.; lucerne chaff (mean of six aliquots) $Na^+ = 8$ (7-10), $K^+ = 595$ (585-615), $Cl^- = 125$ (116-135) m.equiv./kg.

Sheep Adr. 3 was given lucerne chaff alone (0·6 kg./day): the composition was as above.

Sheep Adr. 4 was fed on either chaff alone (0·6 kg./day) or lucerne chaff alone (0·6 kg./day). The composition was the same as for Adr. 1.

Sheep P.F.20 was given 0·4 kg. of oaten chaff and 0·2 kg. of lucerne chaff per day. The composition was—oaten chaff (mean of three aliquots) $Na^+ = 41$ (range —), $K^+ = 308$ (305-310), $Cl^- = 116$ (114-118) m.equiv./kg.; lucerne chaff (mean of six aliquots) $Na^+ = 35$ (34-36), $K^+ = 554$ (535-585), $Cl^- = 192$ (191-194) m.equiv./kg.

The water content of the chaff foods was approximately 10 p.c.

Na+ Intake. The sheep with a unilateral parotid fistula (Adr. 1 and P.F.20) were given, in addition, daily supplements of 25 gm. $NaHCO_3$ (298 m.equiv. Na^+) per rumen tube. Adr. 1 received 70 m.equiv. of Na^+/day in food, whereas P.F.20 received only 23 m.equiv./day.

Drugs. Desoxycorticosterone acetate (Percorten, CIBA) and Cortisone (Cortone, Boots) were used. These were given by intramuscular injection.

Chemical Methods. See Denton, 1956; Denton, Wynn, McDonald and Simon, 1951.

Operation Histories.

Adrenalectomy No. 1. Crossbred Merino ewe, aged 4 years. 27th July, 1954; right adrenalectomy. 6th August, 1954: right parotid fistula (Pavlov-Glinski type). 24th August, 1954: left adrenalectomy.

Parotid Fistula No. 20. Crossbred Merino wether, aged 5 years. 7th June, 1955. Left parotid fistula (Wright type). Left carotid loop. 5th July, 1955: right adrenalectomy. 10th July, 1955: left adrenalectomy.

Adrenalectomy No. 3. Crossbred Merino wether, aged 4 years. 8th October, 1954: right adrenalectomy (pleura opened). The animal did not revive rapidly owing to pneumothorax. 13th October, 1954: air aspirated from pleural cavity. The animal's condition improved during the next 3 days. 9th December, 1954: left parotid fistula made by duct transection technique (Ugolev, 1953). Left carotid loop prepared. March, 1955: fistula closed spontaneously. The animal, however, remained in good condition. 7th April, 1955: left adrenalectomy.

Adrenalectomy No. 4. Crossbred Merino ewe, aged 3 years. 24th February, 1955: right adrenalectomy, right nephrectomy. The hilar adrenal vein entered the inferior vena cava at the junction of the latter and the right renal vein. After excision of the adrenal gland and lateral suture of the inferior vena cava, the right renal vein was obstructed at its termination and hence a right nephrectomy was carried out. Right parotid fistula (Wright type). The terminal portion of the parotid duct became necrotic in the first post-operative week and the fistula closed after a further three weeks. The animal, however, remained in good condition. 19th May, 1955: left adrenalectomy.

Parotid Fistula No. 17. Crossbred Merino wether, aged 4 years. 13th October, 1954: left parotid fistula (Wright type). Division of carotid sinus nerve. Preparation of carotid loop. 30th May, 1955: bilateral adrenalectomy.

Parotid Fistula No. 19. Crossbred Merino ewe, aged 5 years. 2nd June, 1955: left parotid fistula (Wright type). Left carotid loop. 28th June, 1955: bilateral adrenalectomy.

RESULTS.

Maintenance of Adrenalectomized Animals.

Hormone Requirements.

It was found that daily injection of 25 mg. of cortisone and 5-10 mg. of DOCA maintained the animals in good condition, provided Na^+ intake was adequate to cover Na^+ loss in the animals with parotid fistulae. As indicated below, DOCA was the more critical factor. It was found that hormone dosage had to be based on comprehensive biochemical observation during the first post-operative weeks, especially in the sheep with fistulae. Two sheep, P.F.17

and P.F.19, died in the first post-operative week. The former was inadequately prepared for surgery and the latter had arterial plasma biochemical changes which indicated overdosage with adrenal hormones and $NaHCO_3^-$ (pH = 7·55; $Na^+ = 152$; $K^+ = 1·5$; $Cl^- = 90$; $HCO_3^- = 45$ m.equiv./litre). The remainder recovered well after adrenalectomy.

Criteria of normality.

Weight. Apart from short-term fluctuations in weight due to experiments, the animals lost little weight after adrenalectomy. The Table below compares the weight of the animal after it had passed the immediate post-operative phase with that found during the last period under control conditions before death (Table 1).

TABLE 1.

Animal	Immediate post-operative weight kg.	Weight in last control period before death kg.	Duration of maintenance
Adr 1	26·9	26·8	6 months
Adr. 3	33·8	33·9	6 weeks
Adr. 4	28·7	27·3	5 months
P.F. 20	38·5	37·2	7 months

Electrolyte regulation. Table 2 shows the mean and standard deviations of a number of observations on the electrolyte composition of plasma, urine and saliva whilst the sheep were on maintenance dosage of hormones.

Behaviour and appearance. The sheep were active and ate and drank in the usual way.

Completeness of adrenalectomy.

Withdrawal of hormone supplement caused in all animals unequivocal evidence of adrenal insufficiency within 1-2 days after the control period ended (i.e. 2-3 days after the previous hormone injection). After 3-4 days it was clear that the animals would die if the hormones were not replaced.

At the conclusion of the experiments, a post-mortem examination was made of all animals. No adrenal tissue was found at the site of operation, on the posterior abdominal wall, or in the pelvis. All glandular structures in the area which were examined histologically were found to be lymph glands.

The effect of withdrawal of adrenal hormones in adrenalectomized sheep.

In all sheep, changes were observed characteristic of adrenal insufficiency in other species. There was a fall in plasma Na^+ concentration and a rise in K^+ concentration within 2 days (Table 4). The urinary excretion of Na^+ was greatly increased also within 2 days, but an increase in urine volume occurred

TABLE 2.

The composition of plasma, saliva, urine and faeces of adrenalectomized sheep on maintenance dosage of hormone. The mean and standard deviation are given, and the figures in brackets indicate the number of observations.

Sheep (Hormone dosage)		Na^+ m.equiv./l. (no. observations)		K^+ m.equiv./l. (no. observations)		Cl^- m.equiv./l. (no. observations)	
Parotid Fistula No. 20	Plasma	142±2·6	(11)	5·0±0·45	(11)	111±4·2	(11)
25 mg. cortisone							
5-10 mg. DOCA	Saliva	167±4·6	(12)	24·5±4·0	(12)	9±1·2	(12)
	Urine	5·5±4·4	(15)	132±76	(15)	65±42	(15)
	Faeces (m.equiv./kg.)	10±2·9	(8)	47±13·8	(8)	16±4·2	(8)
Adrenalectomy No. 1	Plasma	144·5±3·5	(17)	5·4±0·6	(17)	107±4·0	(17)
25 mg. cortisone							
5 mg. DOCA	Saliva	179±4·7	(13)	17±4·3	(13)	16±3·2	(13)
	Urine	29±19·6	(11)	169±49	(11)	69±24·1	(11)
	Faeces (m.equiv./kg.)	18±4·9	(5)	38±6·5	(5)	11±3·9	(5)
Adrenalectomy No. 4	Plasma	146±1·3	(5)	5·2±0·8	(5)	111±1·8	(5)
25 mg. cortisone							
5 mg. DOCA	Urine	59±23	(8)	205±107	(8)	123±47	(8)
Adrenalectomy No. 3	Plasma	143	(2)	4·6	(2)	117	(2)
25 mg. cortisone							
5 mg. DOCA	Urine	100	(2)	131	(2)	70	(2)
	Faeces (m.equiv./kg.)	12	(1)	46	(1)	18	(1)

TABLE 3.

The effect of withdrawal of adrenal hormones on the urine, the saliva, the Na^+ balance and the weight of adrenalectomized sheep.

Sheep	Day	Total electrolyte intake/day		Hormone supplement		Saliva				Urine				Na^+ balance (m. equiv)	Weight (kg.)
		Na^+	K^+	DOCA	Cortisone	Vol. (l.)	Na^+	K^+	Na^+:K^+	Vol. (l.)	Na^+	K^+	Cl^-		
		(m.equiv.)		(mg.)			(m.equiv./l.)				(m.equiv./l.)				
Adr. 1	0	368	318	5	25	2·11	173	18	9·6	1·24	5	134	93		25·8
	1	368	318	—	—	2·07	171	10	17·1	2·54	7	121	76		
	2	368	318	—	—	1·08	181	12	15·1	3·04	83	68	54		
	3	341	192	—	—	0·49	192	15	12·8	1·66	143	68	66	−136	25·4
Adr. 1	0	368	318	5	25	1·60	173	21	8·2	0·84	33	201	88		26·1
	1	368	318	—	25	2·05	172	13	13·2	2·45	107	84	94	−290	25·0
	2	305	32	—	25	0·97				2·05					
P.F.20	0	321	234	10	25	2·11	166	26	6·4	0·78	6	121	62		39·6
	1	321	234	—	25	1·3	184	13	14·2	4·04	62	52	49		
	2	321	234	—	25	1·2	183	11	16·6	2·00	108	93	58	−310	37·5
Adr. 3	0	5	357	5	25	No fistula				1·15	16	106	61		33·5
	1	5	357	—	—					2·53	41	262	73		
	2	4	298	—	—					1·92	163	153	119		
	3	—	30	—	—					0·35	121	101	154		
	4	—	—	—	—					0·24	62	119	106	−489	30·1

TABLE 4.

The effect of withdrawal of adrenal hormones on the blood of adrenalectomized sheep.

Sheep	Hormone supplement (mg.)		Days after withdrawal	Whole blood pH	Plasma electrolyte concentration (m.equiv./l.)			
	DOCA	Cortisone			Na^+	K^+	Cl^-	HCO_3^-
Control P.F. 1 (non-adrenec.) 20 observations venous blood	—	—	—	—	148±1·6	4·7±0·25	111±3·1	24·3±1·4
Controls P.F. 13 P.F. 17 (non-adrenec.) 15 observations arterial blood	—	—	—	7·36±0·06	147±3·7	4·4±0·38	113±3·5	23·6±2·5
Adr. 1 (venous blood)	5	25	—	7·36	141	5·2	113	22·4
	—	—	1	7·37	142	5·3	110	22·4
	—	—	2	7·41	137	6·4	106	19·2
	—	—	3	7·32	127	9·0	97	19
Adr. 1 (venous blood)	5	25	—	7·27	145	5·3	110	21·7
	—	25	2	7·21	128	7·5	100	19·4
Adr. 1 (venous blood)	5	25	—	7·35	142	5·8	109	22·3
	5	—	1	7·20	138	5·9	104	24
	5	—	2	7·19	140	6·1	106	20·5
	5	—	3	7·30	137	5·3	107	22
	5	—	4	7·22	137	5·7	105	21·8
	5	—	7	7·32	135	5·7	106	22
Adr. 3 (arterial blood)	5	25	—	7·40	146	4·6	114	25·9
	—	—	1	7·30	140	5·2	112	24·8
	—	—	2	7·31	129	6·5	102	19·6
	—	—	3	7·32	132	6·3	101	18·5
	—	—	4	7·26	125	6·6	106	17·8
Adr. 4 (venous blood)	5	25	—	7·31	152	4·9	111	27·6
	—	25	1	7·34	142	5·4	110	24·8
	—	25	2	7·29	138	7·1	112	21·2
	—	25	3	7·23	138	8·1	112	18·8
Adr. 4 (venous blood)	5	25	—	7·35	148	4·4	113	25·8
	5	—	3	—	141	4·9	113	—
	5	—	6	7·38	145	4·0	107	32·1
P.F. 20	10	25	—	7·41	149	5·3	113	23
	—	25	1	7·48	139	6·0	112	23·4
	—	25	2	7·37	137	6·2	102	19

before the excretion of urinary Na^+ reached the maximum (Table 3). In each of three experiments, the salivary $Na^+ : K^+$ ratio rose. The salivary K^+ concentration fell in each experiment, whereas the Na^+ concentration remained constant in one (Adr. 1, DOCA withdrawal) and rose in the other two.

Sheep without a parotid fistula.

Withdrawal of both cortisone and DOCA. Three such experiments were carried out, one on sheep Adr. 3 and two on Adr. 4. Changes in blood, urine and faeces typical of adrenal insufficiency occurred within 36 hours. These were essentially the same as those seen in the sheep with fistulae (Tables 3 and 4), except that Adr. 4 did not have a large increase in urine flow. This animal, however, had the right kidney removed in the course of the right adrenalectomy.

Withdrawal of DOCA alone. One experiment was carried out on Adr. 4. There was no diuresis. Adrenal insufficiency as shown by plasma and urine estimation occurred just as rapidly as when both cortisone and DOCA were withdrawn.

Withdrawal of cortisone alone. Cortisone alone was withdrawn from Adr. 4 on one occasion for 6 days. There was no diuresis. The plasma Na^+ concentration fell from 148·5 to 135 m.equiv./l., but K^+, Cl^-, HCO_3^- and pH were normal. The animal appeared lively and well, and ate its food until the sixth day, when its appetite declined and it became less active.

Sheep with a parotid fistula.

Withdrawal of DOCA alone. This was performed three times, once on Adr. 1 and twice on P.F.20. In these experiments, adrenal insufficiency occurred just as rapidly, and the changes in electrolyte concentration of body fluids were of the same severity as when cortisone was also withheld (Tables 3 and 4).

Withdrawal of both cortisone and DOCA. This was performed on two occasions, once with P.F.20 and once with Adr. 1. A diuresis occurred within 24-48 hours in each experiment (Table 3).

A typical experiment on Adr. 1 will be described in detail (Figs. 1 and 2, also first experiment Table 3), and relevant comparison made with other sheep. This sheep had a small sodium deficit (84 m.equiv.) during the 7 days prior to hormone withdrawal. Throughout the experiment it was maintained on its normal diet, which provided an intake of 70 m.equiv.of Na^+/day, and was given a supplement of 25 gm. (c. 300 m.equiv.) $NaHCO_3$ daily by rumen tube. During the control period there was a positive balance of 68 m.equiv. of K^+ and 53 m.equiv. of Cl^-. Prior to withdrawal of hormone, the animal was maintained by daily injections of cortisone (25 mg.), and DOCA (5 mg.). Both DOCA and cortisone were withdrawn for a period of 3 days.

The following effects were observed (Figs. 1 and 2):

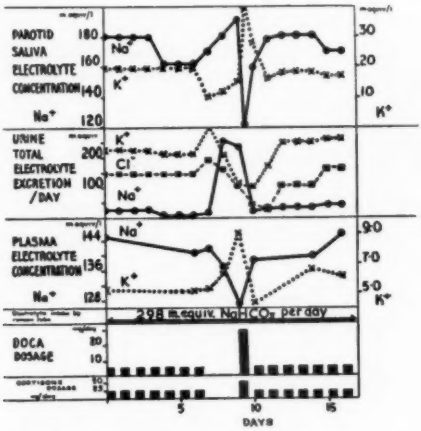

Fig. 1. Sheep Adr. 1. The effect on parotid saliva, urine and plasma of withdrawal of DOCA and cortisone from a bilaterally adrenalectomized sheep with a parotid fistula. The daily Na^+ supplement was given throughout the experiment.

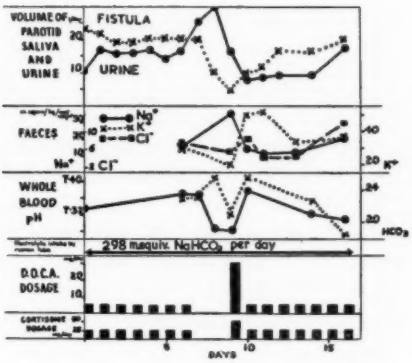

Fig. 2. Sheep Adr. 1. The effect of adrenal insufficiency on the volume of saliva and urine, the faecal composition and the whole blood pH and plasma HCO_3^-. Same experiment as Fig. 1.

Blood and plasma. On the morning of hormone withdrawal, the whole blood pH was 7·36 and plasma Na^+ was 141; K^+, 5·2; Cl^-, 113; HCO_3^-, 22·4 m.equiv./l. No significant change had occurred 24 hours later, but after 48 hours blood pH was 7·41; plasma Na^+, 137; K^+, 6·4; Cl^-, 106; HCO_3^-, 19·2 m.equiv./l. By the following day the whole blood pH was 7·32; plasma Na^+, 127; K^+, 9·0; Cl^-, 97; HCO_3^-, 19 m.equiv./l.

The fall in plasma Na^+ concentration was of the same order as seen in the other experiments recorded in Table 3, but the rise of K^+ concentration was greater.

Urine. During the first 24 hours a profuse diuresis was observed (Fig. 2 and Table 3). The urine volume remained high on the second day, and returned to that seen in the control period by the third day of withdrawal. However, the daily output of Na^+ in the urine was little changed for the first 24 hours of hormone withdrawal but it rose abruptly during the next 2 days. There was an initial increase of K^+ excretion, but it had fallen below normal by the third day.

Saliva. The secretion rate rapidly decreased. The concentration of Na^+ rose steadily from 162 m.equiv./l. at the end of the control period to 192

m.equiv./l. three days later. The concentration of K^+ fell from 20 m.equiv./l. to 15 m.equiv./l., and the $Na^+ : Ka^+$ ratio rose from $162/20 = 8{\cdot}1$ to $192/15 = 12{\cdot}8$.

Faeces. There was an abrupt rise in total Na^+ excretion/day and a fall in K^+ excretion.

Physical state. The sheep appeared normal for the first day. On the morning of the second day it regurgitated when a tube was passed into the rumen, but no other change was observed throughout the second day of hormone withdrawal. However, after 2 days' withdrawal it became rapidly weaker, developed muscle twitching, and either remained sitting in the cage or stood with difficulty. The cardiac rate was regular and normal (72-84 beats/min.) until 62 hours after hormone withdrawal. The rate then suddenly halved. By the end of the third day the sheep was vomiting, hiccoughing, and lying down, and its muscles were flaccid and fibrillating.

The animal ate all its food for the first 2 days, but left 315 mg. during the third day.

Balance studies. Over the 3 days there was a Na^+ deficit of 136 m.equiv. There was a gain of 55 m.equiv. of K^+, and a deficit of 82 m.equiv. of Cl^-. In other experiments (Table 3) there was a greater Na^+ deficit. Voluntary drinking increased from $3{\cdot}2$ to $3{\cdot}8$ litres/day. There was little loss of weight ($0{\cdot}4$ kg.).

The effect of hormone replacement after withdrawal.

At the end of the third day, the sheep was given 50 mg. of cortisone and 30 mg. DOCA. Thereafter it was given 25 mg. cortisone and 5 mg. DOCA daily (Figs. 1 and 2). The $NaHCO_3$ supplement was not given until 3½ hours after the hormone replacement. The following effects were noted:

Blood. On the day after hormone replacement the whole blood pH was $7{\cdot}41$; plasma Na^+, 139; K^+, $4{\cdot}3$; Cl^-, 98; HCO_3^-, $23{\cdot}8$ m.equiv./l.

Urine. The volume remained low for 5 days after hormone replacement. The excretion of Na^+ fell to normal in 24 hours, while that of K^+ rose above normal after 3 days of hormone replacement.

Saliva. There was a large fall of $Na^+ : K^+$ ratio to $121:39 = 3{\cdot}1$ within 2 hours of hormone replacement. The composition of the saliva returned to normal over the ensuing 48 hours. The saliva volume also returned to normal in this time.

A more detailed study of this effect on saliva composition was made on sheep P.F.20, and is described at the end of this section.

Faeces. Na^+ excretion fell to the control value in 24 hours, while the K^+ excretion rose above the control value for the first 2 days of hormone replacement.

Physical state. The animal continued to hiccough for about 4 hours, and then licked the bottom of the cage for several hours. After 4 hours the cardiac rate suddenly rose from 42 to 86 beats/min. and remained normal thereafter, except for a short period of tachycardia (102-112 beats/min.) on the following day. The muscle twitching was still apparent after 24 hours. The animal did not eat all its food until 3 days after hormone replacement.

Balance studies. During the 7 days following hormone replacement, the animal gained 189 m.equiv. of Na^+, lost 156 m.equiv. of K^+, and gained 154 m.equiv. of Cl^-. Voluntary drinking averaged 3·0 litres. There was a weight gain of 0·9 kg. over the period.

The rapidity of action of injected DOCA.

The following experiment was carried out in order to determine the rate and extent of fall of salivary $Na^+ : K^+$ ratio produced by a single intramuscular injection of DOCA (Fig. 3).

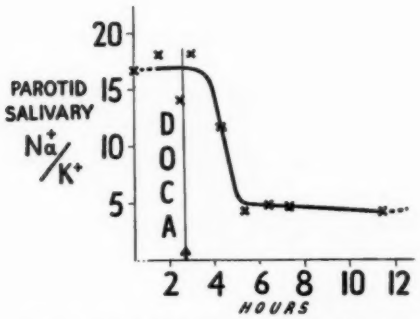

Fig. 3. Sheep P.F.20. The effect of a single intramuscular injection of 20mg. of DOCA on the $Na^+ : K^+$ ratio of the parotid saliva when the animal was in a state of adrenal insufficiency.

The sheep, P.F.20, had been brought to a state of adrenal insufficiency by withdrawal of DOCA for a period of 2 days (Experiment 3, Table 3). It had been given cortisone (25 mg./day) during this period, and its usual diet and daily supplement of 25 gm. $NaHCO_3$ per rumen tube.

On the morning of the third day, the $Na^+ : K^+$ ratio of the overnight collection of saliva was 193/11 = 17·5. It was given 25 mg. cortisone by intramuscular

injection, and 25 gm. $NaHCO_3$ per rumen tube. Collections of saliva were made from a stainless steel tray inserted into the anterior compartment of the metabolism cage. The sheep was not fed until the conclusion of the experiment 11 hours later. After 100 minutes in which two control collections of saliva were made, the sheep was given 20 mg. of DOCA by intramuscular injection. Collections of saliva were made at hourly intervals for the ensuing 5 hours, and a further collection was made 10 hours after the injection of DOCA (Fig. 3).

The $Na^+ : K^+$ ratio of the saliva commenced to fall 2 hours after the administration of DOCA, and reached a minimum of $130:29 = 4{\cdot}5$ after a further hour. It then remained constant for the following 6 hours, but had risen again by the following morning.

Withdrawal of cortisone alone.

This was performed once on sheep Adr. 1 for a period of 7 days. The sheep was given its normal diet, a daily supplement of 300 m.equiv. of Na^+, and 5 mg. DOCA/day.

The following results were obtained:

Blood. There was a gradual fall in plasma Na^+ concentration from 142 to 135 m.equiv./l., but no change in the concentrations of K^+, Cl^- or HCO_3^- in the plasma, or the pH of whole blood.

Urine. There was no significant change in volume or composition of the urine.

Saliva. During the first 5 days, the volume was unchanged at 2·0-2·3 l./day. In the last 2 days of the experiment, the volume fell and was 1·37 litres on the last day. There was little change in $Na^+ : K^+$ ratio of the saliva over the whole period.

Behaviour and appearance. By the end of the 6th day, the animal commenced to leave food in the bin. It still appeared active at the end of the period.

Weight. The animal's weight remained constant over the period.

The effect of administration of adrenal hormones in excess of basal maintenance dosage.

Five such experiments were conducted, four on P.F. 20 and one on Adr. 1. The animals were fed on the diets described in the "Methods" section. In the experiment using Adr. 1, the DOCA supplement was increased to 40 mg. daily, while the daily $NaHCO_3$ supplement was maintained constant at 25 gm. (*c.* 300 m.equiv.). P.F.20 was given 20 mg. DOCA and 25 gm. $NaHCO_3$ daily in each of three experiments, and in a fourth experiment the same supplement of DOCA was given while the daily $NaHCO_3$ supplement was 35 gm. (*c.* 420 m.equiv.). The results (Table 5) were as follows:

TABLE 5.

The effect of excess DOCA administration on the salivary volume and composition, electrolyte balance and weight of adrenalectomized sheep.

Sheep	DOCA (mg./day)	$NaHCO_3$ (m.equiv./day)	Day No.	Saliva				Balance at end of period			Weight (kg.)
				Mean vol. for period (l.)	Composition						
					Na^+	K^+	$Na^+:K^+$	Na^+	K^+	Cl^-	
					(m.equiv./l.)			(m.equiv.)			
Adr. 1	10	300	Control (7 days)	1·54	171	24	7·1				26·8
	40		4	1·98	125	65	1·9	+378	+373	+314	28·8
P.F.20	5	300	Control (4 days)	1·75	172	30	5·7				37·7
	20		6	1·96	150	54	2·8	+126	+1	+85	39·6
P.F.20	10	300	Control (3 days)	1·86	173	34·5	5·0				39·2
	20		5	2·37	136	58	2·3				
	20		7	2·44	138	58	2·4	−76	+355	+186	40·0
P.F.20	5	300	Control (6 days)	1·8	175	29	6·0				37·4
	20		5	2·06	141	58	2·4				
	20		9	2·26	138	57	2·4	−56	+228	+142	38·8
P.F.20	5	417	Control (5 days)	1·60	187	14	13·4				37·2
	20		6	2·55	151	48	3·1				
	20		10	2·87	153	42	3·6	+56	+90	+49	38·3

Blood. There was a rise in the plasma $Na^+ : K^+$ ratio from the mean control value of 28·0 (range 26·2-29·8) to a mean of 46·3 (range 38·6-51·0). The plasma sodium concentration usually rose (mean rise = 6·3 m.equiv./l., range — 2 to + 16 m.equiv./l.) while the K^+ concentration fell in every case (mean fall = 1·8 m.equiv./l., range = 1·1 to 2·5 m.equiv./l.). The plasma Cl^- rose in every experiment, but there was no constant alteration in whole blood pH or plasma HCO_3^- concentration.

Urine. The urine volume fell in four experiments and was unchanged in the fifth. In each of the four experiments in which the daily Na^+ supplement was 300 m.equiv., the daily urinary excretion of Na^+ fell to 1 m.equiv. within 48 hours. It remained low even though DOCA administration was continued for up to 9 days. In each case, there was a reduction in excretion of K^+ and Cl^-.

In the fifth experiment, in which 35 gm./day of $NaHCO_3$ was given (P.F.20, DOCA 20 mg./day), the urinary excretion of Na^+ fell to 1 m.equiv./day within 48 hours, and remained low (less than 3 m.equiv./day) for the following four days. However, on the 7-10th days the daily excretion of Na^+ was 36, 27, 81, 11 m.equiv. respectively (Fig. 4). In this case, the excretion of K^+ was also considerably reduced, but the daily excretion of Cl^- rose a little in the first 6 days and then rose considerably when the Na^+ excretion rose.

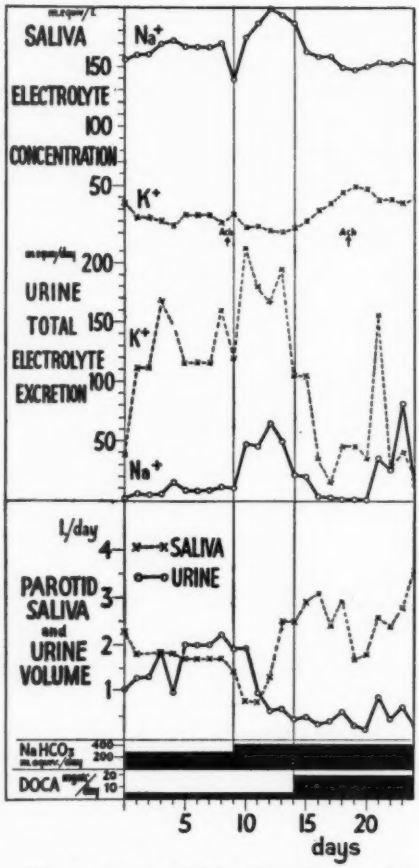

Fig. 4. Sheep P.F.20. The effect of daily injections of 20 mg. DOCA on the volume and electrolyte composition of saliva and urine. The sheep was given 300 m.equiv. $NaHCO_3$ daily in the first control period, then 416 m.equiv. daily for the second control period while on maintenance dosage of DOCA (5 mg./day). Throughout the period of high DOCA dosage, the Na^+ supplement was maintained at 416 m.equiv./day. The cortisone dosage was constant. No recovery period is shown on the graph, as a period of Na^+ depletion followed; the results will be published separately.

Saliva. In the four experiments in which the Na^+ supplement was 300 m.equiv./day, the daily saliva volume increased by 20-50 p.c. 1-2 days after the

DOCA dosage was increased and remained large during the period of increased DOCA dosage (Table 5). In the fifth experiment (P.F.20, DOCA 20 mg./day), the saliva volume rose during the period when the Na^+ supplement was increased to 35 gm. (*c.* 420 m.equiv./day), and remained large throughout the period of increased DOCA dosage (Fig. 4).

In every experiment there was a fall in Na^+ concentration and a rise in K^+ concentration in the saliva. After 4 days an equilibrium was reached and the composition remained constant, even if DOCA administration were continued for 10 days. An increase of the Na^+ supplement caused a rise in $Na^+ : K^+$ ratio of the saliva (Fig. 4, cf. Denton, 1956), whereas DOCA administration caused a fall in this ratio. This is in contrast with the effect on the plasma, where both increasing the Na^+ supplement, and increasing DOCA dosage, caused the $Na^+ : K^+$ ratio to rise.

Faeces. In four experiments, three on P.F.20 and one on Adr. 1, observations were made on the effect of increased DOCA dosage on faecal excretion of electrolytes. In each case, the Na^+ excretion decreased and the K^+ and Cl^- excretion rose. For example, in a typical experiment, Adr. 1 was maintained on cortisone (25 mg.), DOCA (10 mg.), and was given a daily supplement of 25 gm. $NaHCO_3$. The analyses of faeces collected at the end of the control period were Na^+, 17; K^+, 45; Cl^-, 14 m.equiv./kg. Then the sheep was given 40 mg. DOCA/day for 4 days while the cortisone dosage and $NaHCO_3$ supplement were unchanged. Faeces collected at the end of this period had the following composition: Na^+, 8; K^+, 74; Cl^-, 20 m.equiv./kg.

Balance studies (see Table 5). There was a net Na^+ gain in three experiments and a Na^+ deficit in the other two. In each experiment there was a net gain of both K^+ and Cl^-.

The effect of high DOCA dosage under conditions of rapid Na^+ gain.

This experiment was carried out on two occasions, once on Adr. 1 and once on P.F. 20. In the experiment on Adr. 1, the sheep was given a Na^+ supplement of 600 m.equiv./day for 5 days. This amount was greatly in excess of the Na^+ lost from the fistula. The DOCA supplement during this period was 10 mg./day. The DOCA was then increased to 60 mg./day while the Na^+ supplement of 600 m.equiv./day was continued. In the experiment on P.F.20, which was approximately in Na^+ balance at the beginning of the period, the sheep was given a Na^+ supplement of 417 m.equiv./day, and 20 mg. of DOCA/day. However, in this instance, it gained Na^+ rapidly as its saliva volume, and hence Na^+ loss from the fistula, was still low as a result of previous Na^+ and hormone withdrawal experiments.

In each case the animal died without premonitory symptoms. Adr. 1 showed no fall in salivary $Na^+ : K^+$ ratio during the 2 days this regimen was carried out.

DISCUSSION.

It has been shown that adrenalectomized sheep, with or without permanent unilateral parotid fistulae, can be maintained indefinitely in good condition and with constant weight if they are given their ordinary diet and a daily dosage of 25 mg. of cortisone and 5-10 mg. of DOCA. The sheep with parotid fistulae were given a supplementary Na^+ intake adequate to replace the Na^+ lost in the saliva. Analyses of plasma, saliva, urine and faeces of adrenalectomized sheep maintained in this manner deviated a small amount from normal. In the plasma and saliva analyses this may have been accounted for by the fact that the blood was taken 24 hours after the previous hormone injection, and it has been shown by studies of saliva that the action of intramuscularly injected DOCA was maximal 4-5 hours after injection and was clearly diminished 21-24 hours later.

No attempts were made in the course of these studies to determine whether the sheep could be maintained without DOCA if given a high Na^+ and low K^+ diet. The latter would involve an artificial diet, as the animals had a K^+ intake of 230-360 m.equiv./day in their food. However, hormone withdrawal studies, carried out under the standard conditions of Na^+ intake and diet described, indicated that sheep were more sensitive to the withdrawal of DOCA when cortisone intake was continued than man (Lukens, 1952). Withdrawal of DOCA from the adrenalectomized sheep caused a critical state of insufficiency within four days. Indeed, sheep appear to be among the most sensitive of animals to the effects of adrenal insufficiency. The survival time of 7 sheep, which were adrenalectomized in two stages and were given no hormone, was 2-4 days (I. R. McDonald, unpublished).

Adrenal insufficiency in the sheep caused changes in plasma electrolytes similar to those described in other species, e.g. dog (Loeb, Atchley, Benedict and Leland, 1933; Harrop, Soffer, Ellsworth and Trescher, 1933); monkey (Britton, Silvette and Kline, 1938), and man (Hartman and Brownell, 1949).

In the sheep, diuresis occurred within 24 hours of hormone withdrawal and the daily urine volumes were 3-5 times greater than in the control period. In one instance (Adr. 1), this diuresis clearly preceded significant Na^+ loss in the urine by 24 hours and there was no evidence in others that the initial diuresis was osmotically determined. It did not occur on two occasions in Adr. 4, but this sheep had only one kidney (see "Methods") and this may have altered its behaviour, since Verney (personal communication) has shown unilateral nephrectomy to diminish the response to water load in the dog. In the dog, Loeb *et al.* (1933) and Harrop *et al.* (1933) reported an increase in urine volume of approximately 50 p.c. during the first two days of adrenal insufficiency. Subsequently, the urine volume fell. Winter and Hartman (1934) reported that the urinary volume of the adrenally insufficient cat decreased but, as voluntary drinking decreased even further, the animal became depleted of water. In the rat, Rubin and Krick (1936), Beaird and Swann (1937), and Sandberg, Perla and

Holly (1937) demonstrated an initial diuresis after adrenalectomy. This was not large unless the animals were given excess Na^+ in the drinking water, when up to 40 ml./day (normal *c.* 4 ml./day) was reported (Sandberg *et al.*, 1937). Graphs of the effect of hormone withdrawal in adrenalectomized dogs show a similar diuresis (Meier, Gysel and Muller, 1944), but it was not a constant finding and the urine volume closely paralleled Na^+ and Cl^- excretion.

Another feature of interest was the magnitude of the fall of plasma Na^+ concentration relative to alterations in Na^+ balance. In experiments on Na^+ depletion in normal sheep with unilateral parotid fistulae, Na^+ deficits of 500-900 m.equiv. were produced by withholding the $NaHCO_3$ supplement. The plasma Na^+ concentration rarely fell below 135 m.equiv./l. (Denton, 1957). Similar experiments on Na^+ depletion were made on adrenalectomized sheep (Goding and Denton, 1956). These were maintained on a constant but increased DOCA dosage before and during the period of withdrawal of Na^+ supplement. In this way Na^+ deficits of 450-600 m.equiv. were produced, but the plasma Na^+ fell only 1-5 m.equiv./l. As shown here, adrenal insufficiency caused a Na^+ deficit of 136-489 m.equiv., yet in most instances the plasma Na^+ fell below 130 m.equiv./l. This increased lability of plasma Na^+ concentration during adrenal insufficiency compared with that seen in Na^+ depletion from other causes has usually been attributed to the intracellular movement of Na^+. However, as indicated elsewhere (Denton, 1957), there may be an additional reason for this difference *in the sheep*. Because of the dynamics of digestion in the ruminant, which involve the continuous secretion of saliva, the normal animal might have 200-300 m.equiv. of Na^+ in the rumen at any one time (Denton, Wynn, McDonald and Simon, 1951; Denton, 1957). Potentially, this acts as a Na^+ reservoir since, in Na^+ depletion, the $Na^+ : K^+$ ratio of the parotid saliva falls and a fluid of lower Na^+ concentration replaces that absorbed from the rumen in the course of digestion. However, in the particular instance of Na^+ depletion caused by adrenal insufficiency, it has been shown that there is no fall in salivary $Na^+ : K^+$ ratio, and hence the animal cannot draw upon the Na^+ reservoir in the rumen.

Frawley and Thorn (1951) noted that adrenal insufficiency in man was associated with a high $Na^+ : K^+$ ratio in mixed saliva. In the sheep, adrenal insufficiency was associated with a rise in this ratio. This rise occurred in spite of the large Na^+ deficit referred to in the preceding paragraph. Denton (1957) has shown that Na^+ depletion in the normal sheep is associated with a fall in the $Na^+ : K^+$ ratio of the parotid saliva. Hence, it is clear that adrenalectomized sheep which become Na^+ depleted as a result of withdrawal of adrenal hormones are unable to respond as do normal sheep by a fall in $Na^+ : K^+$ ratio.

When DOCA was restored to the sheep after adrenal insufficiency there was a rapid fall in salivary $Na^+ : K^+$ ratio (Figs. 2 and 3). The fall was far greater than that observed after one injection of DOCA while the sheep was in

normal Na^+ balance, and the minimum ratios observed were of the same order as those seen in normal sheep with corresponding degrees of Na^+ depletion. The rapidity of action of the injected DOCA approximates to that of aldosterone on human saliva (Simpson and Tait, 1955).

Administration of DOCA in excess of maintenance dosage led to a rise in the plasma Na^+ concentration and fall in the K^+ concentration typical of other species. Increased DOCA administration in the sheep caused a similar change in parotid salivary $Na^+ : K^+$ ratio to that shown in human mixed saliva by Frawley and Thorn (1951). Mach, Fabre, Duckert, Borth and Ducommun (1954) and Prader, Gautier, Gautier, Naf, Semer and Rothschild (1955) found that aldosterone caused a similar fall in salivary $Na^+ : K^+$ ratio in man. However, the composition of human mixed saliva varies with secretion rate (Prader *et al.*, 1955) and the K^+ concentration is little affected by electrolyte active steroids (Simpson and Tait, 1955). The concentration changes induced by Na^+ depletion (McCance, 1938) and adrenal hormones (Prader *et al.*, 1955) are small (3-5 m.equiv./l.). In the sheep, 1-4 litres of parotid saliva are available each day for analysis. By contrast, the $Na^+ : K^+$ ratio of 24-hour collections of the saliva of a sheep in normal Na^+ balance is little affected by changes of secretion rate (Denton, 1956) while injection of DOCA produces large reciprocal changes of Na^+ and K^+ concentrations (20-60 m.equiv./l.). Thus the sheep would appear to have considerable advantages as an animal for study of adrenal steroids. Furthermore, the effect of adrenal hormone may be determined under circumstances of a precisely defined amount of Na^+ depletion.

The administration of DOCA in excess of maintenance dosage also caused the urinary excretion of Na^+ to fall almost to zero in four out of five experiments. In the fifth experiment, in which the Na^+ intake was increased by 120 m.equiv./day, the excretion of Na^+ rose again after 7 days. This resembles the findings of Conn (1949) and Bass, Kleeman, Quinn, Henschel and Hegnauer (1955). It was observed that, although this "escape" from the effect of DOCA occurred with urinary Na^+ excretion, the $Na^+ : K^+$ ratio of the saliva remained constant until the end of the 10-day period of the experiment.

In the sheep, there was a reduction in urine volume in each experiment in which excess DOCA was administered. This contrasts with the findings of Ragan, Ferrebee, Phyfe, Atchley and Loeb (1940) in which excess DOCA administration in man caused polyuria. Conn (1955) also observed polyuria in a patient with an adrenal tumour producing effects which could be ascribed to excess aldosterone production.

The electrolyte balance studies made during increased DOCA intake showed in two instances a small Na^+ deficit. As faecal and urinary Na^+ excretion were diminished, this resulted from the large increase in saliva volume induced by the DOCA outweighing the conserving effect of lowered salivary Na^+ concentration. In both experiments the expected rise in plasma Na^+ concen-

tration occurred in spite of the Na^+ deficit. Full interpretation of this effect of DOCA cannot be made on the information available. There may have been a redistribution of Na^+ within the body. One possible mechanism of this might lie in the lowered salivary $Na^+ : K^+$ ratio changing the composition of the pool of fluid involved in the dynamics of ruminal digestion, thus making Na^+ available from this reservoir. The K^+ retention observed would be consistent with this. In all instances, increased DOCA dosage caused the animals to gain weight and, as the balances showed a retention of water, the observation of an increase of plasma Na^+ concentration is consistent with the above hypothesis.

SUMMARY.

Bilaterally adrenalectomized sheep can be maintained in good condition indefinitely if given a daily supplement of cortisone (25 mg.) and DOCA (5-10 mg.).

Withdrawal of supplementary hormone intake caused severe adrenal insufficiency within 2-4 days. The plasma $Na^+ : K^+$ fell and there was a large increase in Na^+ loss in the urine and faeces. There was an external Na^+ deficit of 136-489 m.equiv. In sheep with permanent unilateral parotid fistulae, the salivary $Na^+ : K^+$ ratio rose. A striking feature of adrenal insufficiency in the sheep was the large diuresis which occurred during the first 24-48 hours.

The withdrawal of DOCA alone caused changes of a magnitude similar to withdrawal of both DOCA and cortisone. DOCA was the more critical component of the hormone supplement.

Administration of an increased amount of DOCA to the animal caused a rise of plasma $Na^+ : K^+$ ratio and decrease of Na^+ excretion in the urine and faeces. In the animals with parotid fistulae, the salivary $Na^+ : K^+$ ratio fell, and the salivary secretion rate rose by 20-50 p.c. Whereas the salivary $Na^+ : K^+$ remained decreased during 7-10 days of increased DOCA dosage, the urinary Na^+ excretion on one occasion rose again after 7 days.

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THE DETERMINATION OF VITAMIN B_{12}-ACTIVITY IN THE ORGANS AND EXCRETA OF SHEEP

6. VITAMIN B_{12}-ACTIVITY IN PORTAL BLOOD

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The various assay techniques used to measure the vitamin B_{12}-activity in the faeces and the contents of the alimentary canal give widely differing results. On the other hand, the estimate of the vitamin B_{12}-activity in the liver is affected very little by the method of assay (Dawbarn and Hine, 1954, 1955; Hine and Dawbarn, 1954; Dawbarn, Hine and Smith, 1957a). This contrast has been shown by workers at Reading and by others to be due to the presence in gut contents of several factors differing in their vitamin B_{12}-activity for the test microorganisms (Ford and Hutner, 1955).

The present investigation was undertaken to determine whether the multiple factors present in the alimentary canal of the sheep are absorbed into the portal blood stream. Samples of portal and of systemic blood from four sheep were examined, as well as extracts of the liver, rumen contents and small intestine contents.

Measurements of vitamin B_{12}-activity were made by the method of Ford (1953a) using *Ochromonas malhamensis* as the test organism. Ford's method is the best available at the present time for the measurement of true vitamin B_{12}, since *Ochromonas* "shows a metazoan-like specificity in its requirement for 'B_{12}'" (Ford and Hutner, 1955). *Lactobacillus leichmannii* responds to pseudo-vitamin B_{12} and to factor A as well as to vitamin B_{12}. In order to obtain an estimate of these other factors assays were also carried out with *L. leichmannii*.

To ensure a high proportion in the rumen of the factors other than vitamin B_{12}, the sheep were fed for a week or more on a cobalt-deficient diet of wheaten-hay chaff and gluten with a daily supplement of cobalt by mouth. One of the animals, No. 272, had been subsisting on the same ration without the cobalt supplement for many weeks and was losing weight and appetite, typical signs of cobalt deficiency. For seven days before slaughter 1 mg. cobalt was adminis-

tered each day by mouth, with resultant immediate increase in food intake. The other experimental animals were maintained on the supplemented ration for the last three weeks.

METHODS.

Blood samples were collected under anaesthesia into vessels containing heparin, systemic blood from the jugular vein and portal blood from the portal vein close to its entry into the liver. The animals were then killed. The livers and rumen contents were removed from all four. From three the small intestine was also excised and the contents squeezed out. For sheep Nos. 151 and 159 the upper and lower halves were treated separately. The blood samples were centrifuged and the plasmas removed with pasteur pipettes. Liver, gut contents and plasmas were stored at $-20°$ C. prior to assay.

The vitamin B_{12}-active factors were extracted from liver, rumen contents and small intestine contents by adding glass-distilled water containing cyanide, adjusting to pH 5 and steaming for 30 minutes. After cooling, the insoluble residues were removed by centrifuging and/or filtering. The plasmas were treated by the method of Ford (1953b). The vitamin B_{12}-activity was determined in the extracts by the *O. malhamensis* and the *L. leichmannii* methods of assay (Ford, 1953a; Dawbarn *et al.*, 1954, 1957b).

RESULTS AND DISCUSSION.

The results of the assays are presented in Tables 1 and 2.

For three sheep the *L. leichmannii* and *O. malhamensis* estimates of vitamin B_{12}-activity in the liver were the same. In the *L. leichmannii* assay a lack of parallelism between the dose response lines for vitamin B_{12} and for the liver extract of the depleted sheep, No. 272, precluded an accurate determination. The mean value obtained over the whole assay range was slightly lower than that found by the *Ochromonas* assay.

In the contents of the rumen and small intestine the measurements of vitamin B_{12}-activity made by means of the *L. leichmannii* method were from 170 to 330 p.c. of those made by the more specific *Ochromonas* assay.

In the jugular blood plasmas of three of the sheep the two methods of assay gave results which did not differ by more than 3 p.c. It has already been reported that in a series of plasmas obtained from 19 sheep on pasture the vitamin B_{12}-activity measured by the two methods agreed closely (Dawbarn *et al.*, 1957b). Of the factors which are active for *L. leichmannii*, therefore, the systemic blood of these animals seemed to contain only such as are also active for *Ochromonas*. In the portal blood a small amount of some other factor may have been present. The *L. leichmannii* assay gave a 10 p.c. higher value than did the *Ochromonas* assay in the portal plasma of sheep No. 151. For the other two sheep the differences were 5 p.c. and 1 p.c.

In the blood plasma of sheep No. 272, whether systemic or portal, there was a greater discrepancy between the measurements, the *L. leichmannii* assay giving values which were between 20 p.c. and 30 p.c. higher than those of the *Ochromonas* assay. By contrast in the gut contents the difference was over 200 p.c.

TABLE 1.

Vitamin B_{12}-activity in liver, rumen contents and small intestine contents of sheep.

Sheep No.		*L. leichmannii* μg./gm. dry wt. L	*Ochromonas* μg./gm. dry wt. O	L/O × 100
446	Rumen Contents	0·93	0·46	202
	Liver	3·1	3·1	100
151	Rumen Contents	1·38	0·69	200
	Small Intestine Contents			
	Upper	2·35	0·89	264
	Lower	1·46	0·58	252
	Liver	4·8	4·8	100
159	Rumen Contents	1·50	0·88	171
	Small Intestine Contents			
	Upper	3·4	1·85	184
	Lower	3·6	1·86	193
	Liver	5·0	4·9	102
272	Rumen Contents	1·70	0·54	315
	Small Intestine Contents	3·1	0·93	334
	Liver	0·48	0·51	94

TABLE 2.

Vitamin B_{12}-activity in blood plasma of sheep.

Sheep No.	Type of Blood	*L. leichmannii* mμg./ml. L	*Ochromonas* mμg./ml. O	L/O × 100
446	Jugular	1·23	1·27	97
	Portal	1·07	1·06	101
151	Jugular	3·8	3·7	103
	Portal	4·2	3·8	110
159	Jugular	5·6	5·6	100
	Portal	5·9	5·6	105
272	Jugular	1·27	1·04	122
	Portal	1·28	1·01	127

A sample of systemic blood drawn immediately before the first cobalt drench contained 0·44 mμg. vitamin B_{12}-activity per ml. plasma (*L. leichmannii*) compared with 1·27 mμg. per ml. at slaughter 7 days later.

These results might be explained on the assumption that relatively much more of the true vitamin B_{12} in the gut was absorbed into the portal blood than of the other factors. If this were so, it could not be attributed to any difference in the capacity of the various factors to combine with "intrinsic factor" since Gregory and Holdsworth (1953a and b) found that vitamin B_{12}, factor A,

factor B and pseudo-vitamin B_{12} were bound to the same extent by an "intrinsic factor" concentrate from hog stomach and also by a concentrate from sow's whey.

An alternative explanation of the present findings may be put forward. According to this very little absorption of vitamin B_{12}-activity was taking place and the circulating vitamin B_{12} was derived mainly from stores in the tissues. Such an explanation may be reasonable in the case of the three sheep which had adequate stores of the vitamin in the liver and presumably in the other tissues. But the fourth animal was in a seriously depleted condition with vitamin B_{12} in the liver reduced to a fraction of its value in the other animals. The activity in the gut, on the other hand, after one week's oral administration of cobalt, was normal. For this sheep the first suggested explanation seems more likely.

However, a third possibility cannot be ruled out, namely, that all the vitamin B_{12}-active factors were absorbed at a rate depending on their concentration in the small intestine and all but vitamin B_{12} itself were rapidly destroyed or excreted either in the urine or in the faeces. In the course of work on vitamin B_{12} excretion it was shown by comparison of assays and by paper ionophoresis that in the urine of one sheep a considerable proportion of the total vitamin B_{12}-activity was attributable to the other factors. But with the rest of the sheep examined only true vitamin B_{12} could be detected in the urine. Daily injections of 50 μg. vitamin B_{12} were followed by a rise in the vitamin B_{12}-activity in the faeces as well as in the urine (Dawbarn and Hine, 1955).

SUMMARY.

The vitamin B_{12}-activity was measured in extracts of systemic and portal blood plasma, liver, rumen contents and small intestine contents of sheep subsisting on a cobalt-deficient diet of wheaten-hay chaff and gluten and given a cobalt supplement by mouth. The measurements were made by the *Lactobacillus leichmannii* and *Ochromonas malhamensis* assay procedures. Three of the sheep had adequate tissue stores of vitamin B_{12}. The tissues of the fourth animal were seriously depleted but the vitamin B_{12}-activity in the gut contents was normal.

In the livers the two assays gave results which agreed closely. In the contents of rumen and small intestine the measurements made with *L. leichmannii* were 170 to 330 p.c. of those obtained with *O. malhamensis* as the test organism.

The two assays gave the same measure of vitamin B_{12}-activity in the systemic blood plasmas of the sheep with adequate tissue stores of the vitamin. In the portal blood plasmas the *L. leichmannii* estimates averaged 5 p.c. higher.

In the systemic and portal blood plasmas of the depleted sheep the *L. leichmannii* assay gave results which were between 20 p.c. and 30 p.c. higher than those of the *O. malhamensis* assay.

Three possible explanations of the phenomena are suggested.

Acknowledgment. We wish to thank Mr. H. R. Marston, F.R.S., Chief of the Division of Biochemistry and General Nutrition of C.S.I.R.O., for advice and helpful criticism, and Mr. I. G. Jarrett and Mr. B. J. Potter for obtaining the samples of blood, liver, rumen contents and small intestine contents. We are also indebted to Mr. D. W. Dewey who was responsible for the care of the sheep.

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THE DETECTION OF MURRAY VALLEY ENCEPHALITIS ANTIBODIES IN HENS' EGGS

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(*Accepted for publication 26th April, 1957.*)

The current theory concerning the epidemiology of Murray Valley encephalitis (MVE) is that there is a reservoir of the virus, probably avian, in the northern part of Australia (Miles and Dane, 1956; Anderson, 1954). However, so far it has not been possible to detect an area in which the MVE virus is enzootic. The most obvious method of approaching the problem would be to demonstrate the annual development of antibodies capable of neutralizing MVE virus in the sera of birds, man and possibly other animals known to be resident in suspected areas followed by the isolation of MVE virus itself. There are considerable practical difficulties in carrying out a programme of this kind. Firstly, the area to be covered is enormous, and secondly, obtaining, processing and transporting specimens of blood and other material in good condition to appropriate laboratories is very difficult and time-consuming. However, Anderson (1953) showed that it was possible to detect antibodies which neutralize the MVE virus in the yolks of eggs of wild birds, and McLean (1953), in the laboratory, has demonstrated them in hens' eggs. As a result of these findings and of a suggestion from Dr. S. G. Anderson of the Walter and Eliza Hall Institute, it was thought that a very convenient way of detecting the presence of MVE virus would be to obtain eggs from the area in question and test them for neutralizing antibodies.

McLean (1953) tested only three eggs obtained from hens approximately one year old. The hens had previously received two very large loses ($10^6 LD_{50}$) of MVE virus. He also showed that with increasing age chickens showed decreasing susceptibility to the virus. Consequently, before embarking on field work, it was thought wise to determine whether hens of greater age infected with smaller doses of virus would lay eggs containing detectable neutralizing antibodies.

This paper describes experiments which show that neutralizing antibodies may be detected in eggs from hens 2 years old and that the antibodies persist for many months.

Materials and Methods.

Virus: The Adelaide strain of MVE virus passaged intracerebrally in mice was used as a 10 p.c. suspension of infected mouse brain in rabbit serum (10 p.c.) saline. The virus was dispensed in small quantities and stored in a dry ice cabinet.

Animals: Twelve white Leghorn hens aged approximately 2 years were obtained from the Veterinary Division of the Institute.

The mice used in viraemia and neutralization tests were of the Institute strain, aged from three to four weeks.

Experimental Procedure.

The hens were divided into three groups of four each. The first group (hens 1-4) were inoculated with 2·5 mouse LD_{50}, the second (hens 5-8) with 25 LD_{50} and the third (hens 9-12) with 250 LD_{50} of virus.

The hens were bled from a wing vein before inoculation with virus and 1, 2, 3, 4, 7, 15, 21, 28 and 35 days after inoculation and thereafter at 3 to 4 month intervals for a period of 15 months. Up to and including the fifteenth day some of the blood was heparinized and serum obtained from the remainder. After the 15th day only serum was kept.

For approximately six weeks after inoculation the hens were kept in separate cages and the eggs from each hen were identified. Afterwards the hens were run together with two roosters. During this period eggs were collected until more than 14 months after inoculation.

After collection the eggs were broken and the yolks and whites were separated and stored in ¼ oz. screw-capped bottles.

All specimens obtained were stored at $-20°$ C. in a freezer. The heparinized specimens of blood were included among them in error as they were destined for viraemia tests.

Viraemia tests.

Tests for viraemia were carried out by inoculating 0·03 ml. of heparinized blood intracerebrally into groups of 10 mice. The mice were observed for 14 days subsequently for characteristic illness and deaths. All deaths occurring after 48 hours were recorded.

Preparation of egg material for neutralization tests.

(a) Yolks were found to become very glutinous after freezing and they were prepared on the day previous to testing. The yolk was removed from the freezer and allowed to thaw at room temperature. Some of the yolk was then placed in a previously weighed test tube adapted for grinding with a fitted pestle (Griffith's tube). The yolk and tube were reweighed, sufficient saline added to make a 20 p.c. suspension w/v of yolk. Some sterile sand was added and the mixture ground until a homogeneous suspension was obtained. The suspension was allowed to stand overnight in the refrigerator at $+4°$ C. and then centrifuged for 5 minutes at 1,000 r.p.m. in a refrigerated centrifuge. The supernatant fluid was removed and used in the neutralization tests described below.

(b) Egg white was thawed on the day it was used. No special preparation was required and it was used undiluted.

Neutralization tests.

Screening tests: When it was fairly certain that specimens had no neutralizing activity three or four dilutions of virus were prepared so that one contained about 5 to 10 LD_{50} and the others between 10 and 100 LD_{50}. Equal quantities of virus preparation and material being tested for neutralizing activity were mixed and incubated at 37° C. for 2 hours. After incubation, 0·03 ml. quantities were inoculated intracerebrally into each one of 5 mice.

Titrations. (a) *Standard*: Five tenfold serial dilutions of virus suspension were mixed in 0·2 ml. quantities with equal quantities of the material under test. The mixtures were incubated and inoculated as described above. With each batch of titrations a control was carried out using rabbit or preinoculation chicken serum in serum neutralization tests or known antibody-free yolks and whites for egg neutralization tests.

(b) *Modified*: To increase the precision of the standard titration it was modified by using ten instead of five mice for the three middle dilutions and five for the terminal dilutions.

From the results of the titrations the LD_{50} was calculated by the method of Kärber (Finney, 1952) and the results expressed as the negative logarithm. The neutralization index, in each instance, was obtained from the difference between the logarithms of the control and test titrations. The neutralization indices are expressed as logarithms throughout this paper. The neutralization indices of yolks refer to the 20 p.c. suspension and are not corrected for dilution.

RESULTS.

Viraemia.

Table 1 shows the results of the viraemia tests. It is seen that a certain small proportion of deaths occurred as the result of inoculating preinoculation bloods. These deaths occurred early and were not preceded by characteristic illness and, thus, were presumably non-specific, following the inoculation of whole blood.

TABLE 1.

Viraemia in fowls after inoculation with MVE virus.

Dose of virus (LD_{50})	2·5				25				250			
Fowl number	1	2	3	4	5	6	7	8	9	10	11	12
Before inoculation	2/10	2/9	1/10	0/10	0/10	0/9	1/17	1/9	0/10	1/9	0/10	0/8
Day 1	2/7	0/8	0/10	0/9	1/6	0/5	1/7	0/8	1/7	0/6	1/6	0/9
Day 2	1/10	2/10	5/8	2/11	10/10	10/10	10/10	0/10	10/10	6/10	1/7	1/10
Day 3	2/4	4/8	9/9	6/9	8/8	5/6	7/7	0/9	6/6	8/9	6/7	5/5
Day 4	9/10	7/7	7/7	10/10	1/9	4/10	6/11	0/7	0/10	10/11	10/10	9/9
Day 7	1/9	2/10	3/10	1/8	0/9	0/7	3/10	1/10	2/9	1/9	0/10	1/6

Denominator = Number of mice inoculated.
Numerator = Number of deaths occurring after 48 hours.

Otherwise it appears that no marked viraemia occurred before the second day and by the seventh there was little or none. Except for hen No. 8, viraemia was detectable in all birds at one time or another during the second, third or fourth days and lasted at the most for 5 days. However, these specimens were stored for up to two months at — 20° C. which may have resulted in the loss of some virus activity.

Serum neutralizing antibodies.

Preliminary tests of the preinoculation sera by the standard neutralizing titration using rabbit serum as a control showed no neutralizing activity.

The results of tests carried out by the modified titration on sera taken at different times are shown in Table 2. The sera from each fowl were tested in parallel using preinoculation sera as controls.

TABLE 2.

Neutralizing activity of fowl sera after inoculation with MVE virus.

Titre Virus Inoc.	Fowl Number	Weeks after Inoculation					
		1	2	4	5	38	64
2·5 LD_{50}	1	0·2*	2·0	2·0	2·0	2·2	,,
	2	,,	1·6	2·2	2·1	2·0	3·0
	3	0·9	2·8	3·1	3·5	2·8	3·4
	4	0·9	2·7	2·5	2·7	2·5	2·7
25 LD_{50}	5	1·1	2·0	2·4	2·4	2·3	2·3
	6	0·5	1·4	1·7	1·8	2·9	3·0
	7	1·2	2·6	3·0	3·1	3·1	2·8
	8	0·2	2·3	2·2	2·8	3·6	2·7
250 LD_{50}	9	1·1	2·0	1·0	2·1	2·1	3·1
	10	0·9	1·5	2·2	2·2	2·7	2·7
	11	0·2	1·4	2·2	2·2	1·9	3·0
	12	0·3	2·5	1·9	3·1	2·4	2·2

Neutralization tests were carried out by the modified method described in the text using preinoculation sera as controls.

* Logarithm of neutralizing index.

,, = not examined.

Neutralizing antibodies developed in sera from all hens, including No. 8 in which viraemia was not demonstrated. They were first detected two weeks after inoculation and the log. neutralization indices (N.I.) did not seem to vary substantially among the fowls for the 14 months following inoculation. Nor did there seem to be a marked difference between the groups receiving different doses of virus.

From the results obtained in the second week and after, the highest log. N.I. was 3·4 and the lowest 1·0, the mean (geometric) being 2·42.

Comparison of neutralizing activity of whites and yolks.

Table 3 shows the results of comparing the neutralizing activities of whites and yolks of the same six eggs by the standard titration. It is seen that the yolks show higher log. N.I. in all but one instance where the neutralizing activity is equal in both. Thus although neutralizing activity is present in the white it is on the average higher in yolk diluted 1 : 5 in saline.

TABLE 3.

Comparison of neutralizing activity from yolks and whites from the same eggs.

Egg Number	White	Yolk
W 1·1	1·0*	2·6
W 1·2	1·8	2·4
W 1·3	1·6	1·6
W 1·4	1·0	1·6
W 2·2	0·8	1·8
W 2·3	1·4	2·6

Neutralizing activity was determined by the "Standard" titration. Yolks and whites known not to contain antibodies were used as controls.

* Logarithm of neutralizing index.

Neutralizing activity in yolks.

Table 4 shows the results of neutralization tests on yolks from individual birds. Twelve yolks from the eggs of seven hens laid before inoculation or within 8 days of it showed no neutralizing activity as determined by screening tests. As determined by the modified titration, those yolks obtained about three weeks to one month after inoculation had, on the whole, less neutralizing activity than those obtained five or more weeks afterwards. Eggs from all the hens showed some neutralizing activity at one time or other.

TABLE 4.

Neutralizing activity of yolks from individual hens after inoculation with MVE virus.

Dose Virus Inoc.	Fowl Numbers	Days after inoculation			
		−2 to +8	21 to 23	27 to 31	37
2·5 LD_{50}	1	,,	,,	1·0*	,,
	2	−(2)[1]	1·7[2]	,,	2·3
	3	−	,,	1·3*	,,
	4	−	1·5	,,	2·3
25 LD_{50}	5	−(2)	,,	1·1*	,,
	6	,,	,,	1·1*	,,
	7	,,	2·6	,,	2·0
	8	,,	1·2	,,	2·3
250 LD_{50}	9	−(2)	2·2	,,	2·3
	10	−(2)	,,	0·6*	,,
	11	−(2)	1·6	,,	1·4*
	12	,,	,,	0·6*	,,

[1] Figures in brackets are the number of yolks tested.

[2] Logarithm of neutralization index.

− = Negative in screening test.

The log. neutralizing indices were obtained from "modified" titrations.

* These results obtained in a separate experiment from others with yolks stored 15 months, all other yolks stored 10 months.

,, = not examined.

Table 5 shows the results obtained from the modified titration of yolks obtained at different periods after inoculation. Yolks collected at two months, about 11 months and about 14 months after inoculation all showed detectable neutralizing activity. All those collected at five weeks and thereafter showed log. N.I. of approximately the same order as one another, the highest recorded being 2·6 and the lowest 1·6. The log. of the geometric mean N.I. was 2·00, little less than for the serum neutralizing antibodies.

Yolks obtained five weeks or more after inoculation had been stored for varying periods ranging from one to ten months and there was no marked difference in log. N.I. between those stored for long and short periods.

TABLE 5.

Neutralizing activity of yolks from hens at intervals after inoculation with MVE virus.

Months after inoculation	No. of yolks tested	Log. neutralization indices
2	6	1·6, 2·1, 1·9, 1·9, 2·0, 2·0
11 to 12	6	1·8, 2·0, 2·3, 2·0, 1·7, 1·8
14 to 15	5	2·6, 2·1, 2·4, 2·2, 2·0

DISCUSSION.

The results described in this paper show that, although there may be increasing resistance with age (McLean, 1953), two-year-old hens are susceptible to infection with relatively small doses of MVE virus. As far as the occurrence of viraemia is concerned, although the blood had not been stored under optimum conditions, the findings are not at variance with those of McLean (1953) for younger birds.

Initial experiments showed that antibodies neutralizing MVE virus were present in the white and yolks of eggs of our hens. Egg white would have been easier to handle, but its neutralizing activity was found to be less than that of yolks. Also there was no evidence that the neutralizing activity of yolks was markedly diminished after storage for many months. Consequently the use of egg white was abandoned.

Infection of our birds was followed in every one by the development of neutralizing antibodies in serum and yolk. This suggests that the same would occur with all fowls younger than two years, which are known to be more susceptible. The antibodies in yolks reach their maximum activity in about five weeks after inoculation, a little later than in the serum. However, the maximum neutralizing activity of the yolks seems to be approximately equal to or very little less than in serum. The antibodies from both sources persist for at least 14 months without marked alteration in activity. Thus it seems that the antibodies found in egg yolks reflect those in the serum of the hens laying the eggs.

McLean (1953) observed that the neutralization indices in sera from older

hens were not very high. Our findings confirmed this and as a result we decided to increase the precision of our neutralization test by increasing the number of animals used for critical dilutions so that there would be less chance of yolks with relatively low neutralizing activity being recorded as having none. It seems that it would probably be valuable to use a test of greater precision for field work where both samples and the incidence of neutralizing antibodies in them may be small.

In view of our findings and those of Anderson (1954) and McLean (1953), it seems that the use of eggs is ideally suited to the study of the epidemiology of MVE. It requires no special skill to collect eggs and they may be relatively easily transported to a laboratory and it is fairly certain that the yolks will be sterile. Anderson (1953), Anderson and Eagle (1953), and Anderson, White and McLean (1954), and Miles and Dane (1956) have shown the value of the detection of antibodies in the sera of domestic fowls of known age for detecting the presence of the MVE virus in certain areas. Since the antibodies in yolk appear to reflect the condition of the serum, the same information may be obtained with much greater ease by their use. Using this method it should be possible to cover a wide area of northern Australia and perhaps map out the extent to which the MVE virus spreads.

SUMMARY.

Small doses of MVE virus infected twelve two-year-old hens producing viraemia, in all but one, and serum neutralizing antibodies in all. The antibodies persisted unchanged for more than 14 months. Antibodies of about the same strength occurred in yolks from the eggs of all the hens throughout the same period. Neutralization indices were not markedly high and a method for increasing the certainty of detecting neutralizing antibodies in yolks is described. The results show that the detection of neutralizing antibodies in hens' eggs may be applied to the study of the epidemiology of the MVE virus.

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PROPAGATION IN TISSUE CULTURE OF THE VIRUS OF INFECTIOUS LARYNGOTRACHEITIS OF FOWLS

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Infectious laryngotracheitis (ILT) was recognised as a disease entity of poultry in 1924, and established as a virus disease by Beach (1931). The pathology in chickens was described by Seifried (1931). Burnet (1934) showed that the virus could be grown on the chorio-allantoic membrane of the developing chick embryo and described the histopathology of the infected membrane. The appearance of the lesions may vary somewhat with the strain of virus (Burnet, 1936).

The capacity of the virus to attack and kill the cells of the adult fowl and also those of the chorio-allantoic membrane, suggested that it might be suitable for study in tissue cultures of chick-embryo fibroblasts. Successful cultivation of the virus was reported by Beach (1932), using cell cultures of the Maitland type (Maitland, 1928). The destruction of chick-embryo lung cells grown on a glass surface by centrifuged material prepared from infected chorio-allantoic membranes, was observed by Pulsford (1955). The cells were destroyed slowly (3-7 days), during which time they rounded off, became more intensely stained and the internal structures more difficult to see. No evidence of inclusion bodies was seen.

The work to be described had three objects, firstly, to attempt serial passage of ILT virus in tissue cultures, secondly, to study the effect of virus on the cells, and thirdly, to examine the properties of the virus after serial passage.

Materials and Methods.

One of the viruses used was strain E973 isolated in Queensland in 1953 by Simmons (1954) from cases of sub-acute disease, and maintained since by continued egg passage. Its present virulence for the chorio-allantoic membrane is comparable to that of the original isolate. Seven other strains were tested for inclusion body formation. Strain S was obtained in 1953 from the Walter and Eliza Hall Institute of Medical Research, Melbourne, and since maintained at the Queensland Institute of Medical Research by egg passage. Other strains G.2817/1, G.2817/2, H.2798, H.2184, G.3221 and G.2642 were recently isolated at the Animal Health Laboratory, Brisbane.

Tissue cultures were made by the roller tube method developed by Gey (1933) and the techniques used were, with minor modifications, those described by Weller (1953) and Robbins (1952).

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Fibroblast cultures were prepared using pulped chick-embryo heart muscle as cell inoculum and cultures of respiratory epithelium were prepared using pulped lung from 11-12 day chick embryos. Pulping in both cases was carried out by first mincing the tissue with scissors then passing it repeatedly through a 23-gauge needle mounted on a syringe.

Cultures for histological examination were prepared on coverglasses in roller tubes by the method illustrated by White (1954).

In the early experiments the growth medium consisted of Earle's salt solution (Earle, 1943) 80 p.c., chick-embryo extract 10 p.c., and human serum 10 p.c., plus penicillin 50 units/ml. and streptomycin 50 µg./ml. The pH was adjusted to $7{\cdot}6 \pm 0{\cdot}1$ with sterile 1 p.c. sodium bicarbonate. Chick-embryo extract was prepared by removing nine-day embryos aseptically, dissecting out the eyes, washing to remove blood and forcing through the 1 mm. aperture of a syringe into an equal volume of Earle's solution. The mixture was incubated for 1 hour at 37° C., centrifuged at 3000 r.p.m. for 10 minutes, and the supernatant frozen and thawed twice before use. Fresh human serum was obtained and heated to 56° C. for 30 minutes before use. Fowl plasma was obtained from heparinised whole blood taken by cardiac puncture from a young, healthy, fully grown fowl. The heparin concentration used was 2·5 I.U./ml., and the separated plasma was stored at $-20°$ C.

Later, a medium consisting of lactalbumin hydrolysate 0·5 p.c. and yeast extract 0·1 p.c. in Earle's salt solution to which 20 p.c. inactivated horse serum was added together with penicillin 50 units/ml. and streptomycin 50 µg./ml., was found to be a more satisfactory growth medium for both fibroblasts and epithelial cells. As before, the pH of the medium was adjusted to $7{\cdot}6 \pm 0{\cdot}1$ with sterile 1 p.c. sodium bicarbonate.

Infected chorio-allantoic membranes were ground in a sterile mortar with alundum powder and 3 ml. per membrane of sterile saline for egg inoculation or Earle's solution for tissue culture inoculation. The fluid so obtained was clarified by centrifugation at 3000 r.p.m. for 10 minutes. Tissue cultures were ground by stripping the cell layer off the glass with a platinum spatula, and grinding it with 1 ml. of fluid in a pyrex tube with a specially fitted ground-glass plunger.

Inoculation of fertile eggs was carried out as described by Beveridge and Burnet (1946).

Histological specimens were prepared by removing the coverglass bearing the tissue culture from the roller tube, washing briefly in Ringer's solution, and fixing in Carnoy's fluid. Tissue preparations were stained by Giemsa.

Pyrex glassware was used throughout, and was cleaned by the method of Robbins (1952).

Throughout the work, samples of the growth medium used, virus inocula from ground chorio-allantoic membrane and ground tissue cultures were tested for bacterial sterility.

EXPERIMENTAL.

Cytopathogenic effect.

Fibroblasts. Fibroblast cultures, 4 days old and averaging 5 mm. in diameter, were inoculated with 0·1 ml. of virus suspension, containing approximately 10^3 infective particles as judged by pock counts on chorio-allantoic membranes. The cultures continued to enlarge until the third day after inoculation. On the fifth day, degenerative processes such as retraction of the cell processes, rounding up of the cytoplasm and vacuolation were evident. The nuclei became more compact and took on an intense basophilia. As destruction of the marginal cells progressed, the affected cells became detached from the glass so that on the eighth or tenth day only the original tissue explant remained.

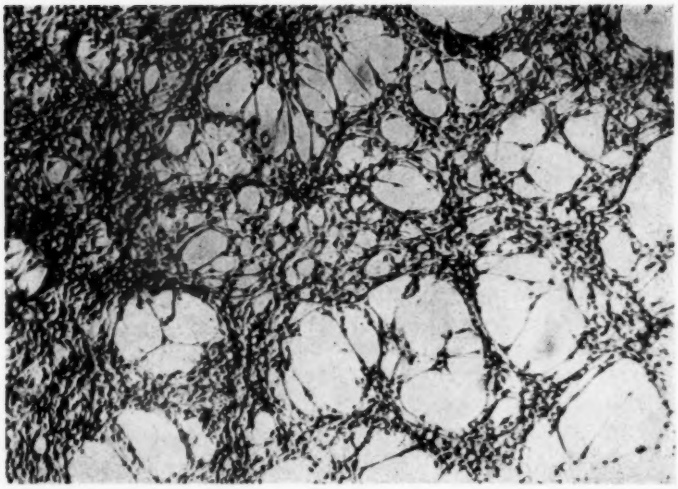

Fig. 1. Normal fibroblasts after 12 days' cultivation. Stain: Haematoxylin and eosin. × 100.

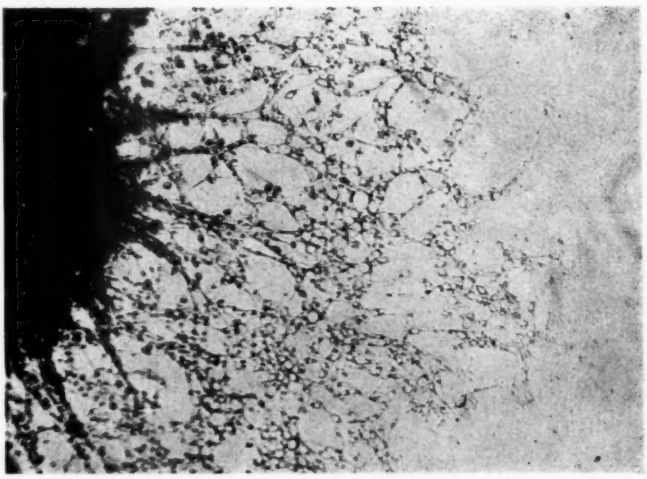

Fig. 2. Fibroblasts 8 days after infection with ILT virus. Original tissue explant is on the left. Strain: Haematoxylin and eosin. × 100.

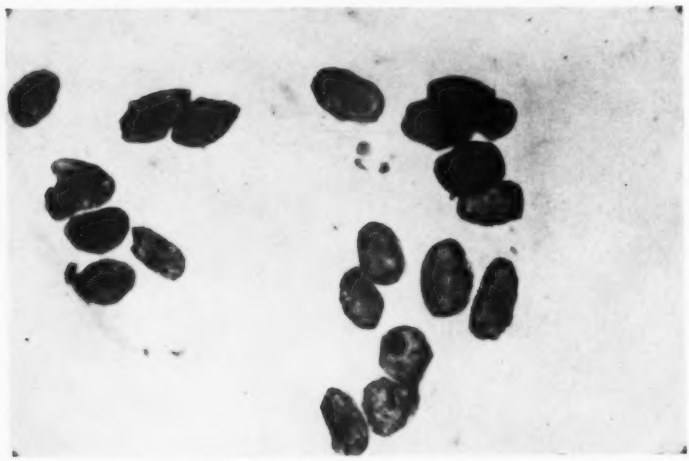

Fig. 3. Cultured respiratory epithelial cells 48 hours after infection with ILT strain H.2184. Several stages in the formation of intranuclear inclusions are shown. Stain: Giemsa. × 1500.

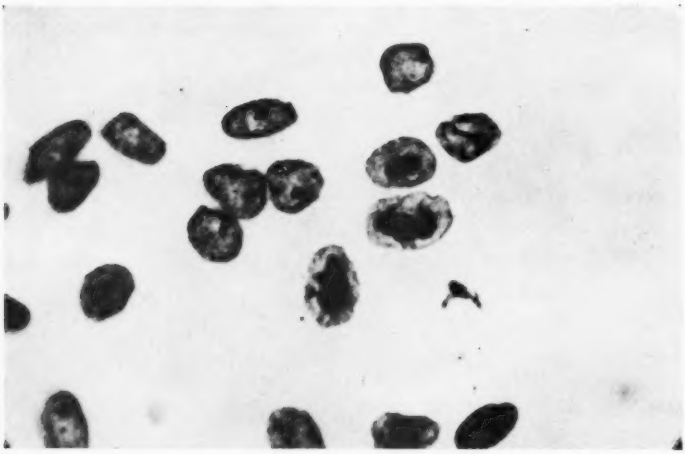

Fig. 4. Cultured respiratory epithelial cells 96 hours after infection with ILT strain H.2184. Three fully mature intranuclear inclusions are shown. Stain: Giemsa. × 1500.

Thirty-five four-day-old cultures were inoculated in three experiments. Destruction of the cells was observed in thirty-one after 5 ± 1 days' further incubation, while uninoculated controls remained healthy.

The changes observed in infected fibroblast cultures are illustrated in Figs. 1 and 2.

Respiratory epithelium. Respiratory epithelium was grown on coverglasses in roller tubes using lactalbumin medium. Growth occurred so that each coverglass was almost completely covered with epithelial cells in 3-4 days. Each culture was inoculated with 0·1 ml. of virus suspension containing approximately 10^3 infective particles.

Infection was evident as early as 18 hours by changes in the cell nuclei leading to the appearance of intra-nuclear inclusions. On the second day of infection cytopathic changes were evident in the cell sheet, an effect which progressed steadily over 2-3 days and resulted in few unaffected cells remaining. Of thirty-eight epithelial cell cultures inoculated thirty-seven showed cytopathic changes, and destruction of the cell sheet was complete in thirty-five of the cultures in 3 ± 1 days while uninoculated controls remained healthy.

Inclusion body formation.

Initial attempts to demonstrate the classical inclusion bodies described by Seifried (1931) and Burnet (1934) using fibroblast cultures infected with E973 were unsuccessful. Cultures grown on coverglasses were removed from the tubes at the time when the cytopathogenic effect first appeared and at later stages up to the time of almost complete destruction of the marginal cells. Although obvious cellular destruction had taken place, no typical inclusion bodies were demonstrated. No inclusion bodies could be demonstrated in chorio-allantoic membranes infected with the E973 strain of virus.

Seven freshly isolated strains were obtained and tested for their ability to produce inclusion bodies in the cells of the chorio-allantoic membrane and to infect the twelve-day chick embryo following amniotic inoculation with the production of inclusions in the cells of the respiratory epithelium. Strain H2184 was selected as the most satisfactory. A series of coverglass cultures of respiratory epithelium was infected with strain H2184 and at intervals of 12 hours beginning 8 hours after infection two cultures were removed, fixed, stained by Giemsa and examined. The cells of uninfected cultures exhibited normal cytology for the whole period of observation (fourteen days).

The first evidence of infection was a bluish green body seen in the region of the nucleolus. At a later stage the body appeared enlarged and stained pink. Subsequently it filled the greater part of the nucleus, pushing the nucleolus to one side while the nuclear membrane showed a marked thickening. The fully mature inclusion appeared as a sharply defined, somewhat irregular body still contained within the degenerating nucleus (see Figs. 3 and 4).

Inclusion bodies were found at all stages during the destruction of the epithelial sheet by the virus, but were most numerous on the second and third day of infection. Cells showing inclusion bodies were often arranged in small groups (see Fig. 3) yet destruction of the sheet appeared to occur more or less uniformly and not by the extension of distinct foci of infection.

Formation of Dulbecco plaques.

Sheets of cells were prepared by the method of Noyes (1953) in 5 cm. Petri dishes for the Dulbecco plaque technique (Dulbecco, 1952). These cultures were then inoculated with dilutions of the ILT virus, and the plates incubated for a further 5 days at 37° C. in 5 p.c. carbon dioxide.

Areas of destruction (plaques) were observed in the cell sheet in numbers varying with the dilution of the inoculum (Table 1). The average diameter of well isolated plaques was 3 mm., although many were asymmetrical.

Plaque counts on sheet cultures of fibroblasts and on respiratory epithelial cells were compared with pock counts on the chorio-allantoic membrane. The plaque counts were significantly higher ($P = 0{\cdot}02$) than the pock counts with the 10^{-1} dilution of virus.

TABLE 1.

Comparison of pock counts on the chorio-allantoic membrane with plaque counts on sheet cultures of chick embryo fibroblasts and chick embryo respiratory epithelial cells.

Virus Dilution	Chorio-allantoic membrane. Pock Counts			Fibroblast sheet. Plaque Counts			Resp. Epithelial Cell Sheet. Plaque Counts		
	Membrane			Culture			Culture		
	1	2	3	1	2	3	1	2	3
Undil.	100	SCL*	SCL	SCL	SCL	SCL	SCL	SCL	SCL
10^{-1}	18	12	15	22	27	23	28	23	26
10^{-2}	1	3	2	5	1	7	4	7	3

* SCL = semi confluent lysis.

Neutralisation of Cytopathogenic Effect by Immune Serum.

The neutralisation of virus by means of serum from an immunised fowl was carried out in parallel by titrations on the chorio-allantoic membrane and in tissue culture, using inocula of 0·05 and 0·1 ml. of virus-serum mixture respectively.

Two series of tissue cultures were used. Series A was inoculated with virus-serum mixtures, and incubated with changes of medium twice weekly. Series B was inoculated in the same way, but antiserum to give a final concentration of 1 in 50 was also incorporated in the culture medium.

From the data in Table 2, it would appear that an inoculum of approximately 10^2 pock-producing doses, as judged from chorio-allantoic membrane

TABLE 2.

The neutralisation of infection of the chorio-allantoic membrane and of the cytopathogenic effect in tissue culture by immune serum.

	Unneutralised												Neutralised											
	Egg				Tissue Culture								Egg				Tissue Culture							
									Time taken after inoculation for cytopathogenic effect to appear (days)								Series A Time taken after inoculation for cytopathogenic effect to appear (days)						Series B Time taken after inoculation for cytopathogenic effect to appear (days)					
Virus Diln.	Pock counts per 0·05 ml.				Controls no virus inoculum								Pock counts per 0·05 ml.											
Egg or Culture No.	1	2	3	4	1	2	3	4	1	2	3	4	5	6	1	2	3	4	1	2	3	4	5	6	1	2	3	4	5	6
No virus					n	n	n	n																						
Undiluted	confluent lesions								5	5	6	6	6	6	confluent lesions				6	6	6	5	6	6	n	13*	n	X	n	n
10^{-1}	confluent lesions								6	6	5	6	6	5	semi-confluent lesions				7	6	6	7	5	6	n	n	n	17*	n	n
10^{-2}	confluent lesions								7	6	X	6	5	6	semi-confluent lesions				8	7	n	6	n	6	n	n	n	X	n	n
10^{-3}	confluent lesions								6	6	7	6	7	7	18	5	11	13	13	n	n	8	n	n	n	n	n	n	n	n
10^{-4}	semi-confluent lesions								7	6	7	6	6	6	—	—	3	1	n	n	15	n	n	X	n	X	n	n	n	n
10^{-5}	43	28	19	31					8	X	7	8	n	n	—	—	—	—	n	n	n	X	n	n	n	n	n	n	X	n
10^{-6}	2	5	3	2					n	n	n	n	n	n	—	—	—	—	n	n	n	n	n	n	n	n	n	n	n	n

n = no cytopathogenic effect seen up to 20th day after inoculation.
X = degeneration of culture other than cytopathogenic effect.
* = evidence of slight vacuolation.
Series A—Growth medium contains no antiserum. Series B—Growth medium contains antiserum 1 in 50.

titrations, was needed to produce a consistent cytopathogenic effect in tissue culture (i.e. 0·1 ml. of a 10^{-4} dilution). Of the five tissue cultures in Series A which received an inoculum of 10^2 pock-producing doses, four show no cytopathogenic effect. Cultures in Series B which received a similar inoculum showed complete suppression of the cytopathogenic effect. The cytopathogenic effect of larger virus inocula was also suppressed.

Serial Passage in Tissue Culture.

Attempts were made to recover the virus from tissue cultures by examining both the fluid phase and the tissue. Five 0·05 ml. samples of the fluid were removed from the cultures 24 hours before the appearance of the cytopathogenic effect, and on the first and third days after it was observed. These were used to inoculate the chorio-allantoic membrane of fertile eggs. In no case did virus lesions appear on the membrane inoculated with this material.

Fibroblast sheets were prepared on the walls of roller tubes by the method of Noyes (1953), infected, and, after incubation for a further six days until the cytopathogenic effect appeared, ground and examined for virus. Four 0·05 ml. samples of this material were inoculated onto chorio-allantoic membranes, and remaining 0·1 ml. samples to further tissue cultures. In this way the virus was successfully passaged through three transfers in tissue culture over sixteen days (Table 3).

TABLE 3.

The passage of the virus of infectious laryngotracheitis in tissue culture.

Passage	Age of culture on inoculation (days)	No. of cultures inoculated	No. showing cytopathogenic effect	Average time taken after inoculation for cytopathogenic effect to appear (days)
1	4	4	4	5±1
2	4	4	3	5±1
3	4	4	3	6±1

Growth Curves.

A series of cultures of respiratory epithelium was set up. To these cultures was added 0·1 ml. of ground chorio-allantoic membrane infected with strain H.2184, to give an initial concentration of *ca.* 10^4 pock-producing doses per ml. of tissue culture fluid. Simultaneously a similar inoculum was made into a series of tubes containing lactalbumin medium but with no tissue.

At intervals, tubes were selected and the whole of the tube contents, fluid plus tissue, was added to a tissue grinder. After grinding, virus was assayed by pooling the homogenate from three tubes and inoculating eggs in duplicate with suitable dilutions of this material.

It can be seen that in medium alone without tissue the virus disappeared before 24 hours. Absorption by the tissue, however, caused the disappearance of virus in less than 12 hours. Reappearance was noted at 24 hours although the large sampling interval may not have allowed the detection of first virus liberated. Maximum titre of $10^{5.8}$ pock-producing doses per ml. was reached at 48 hours. Appreciable virus was detected at 96 hours. At this time the cytopathogenicity had resulted in destruction of most of the cells of the tissue.

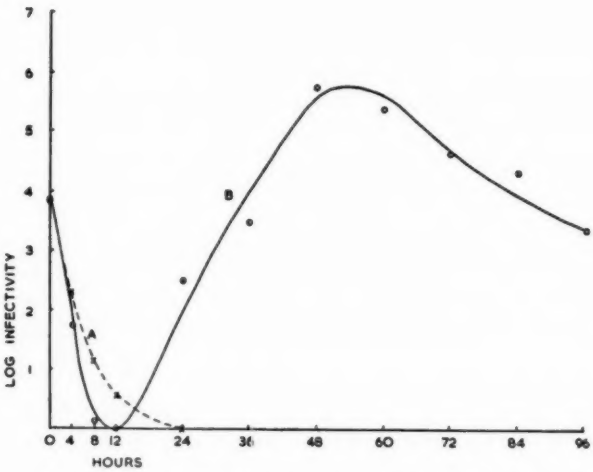

Fig. 5. Growth of Infectious Laryngotracheitis Virus strain H.2184 in Tissue Culture.

Curve A shows the rate of inactivation of virus in lactalbumin medium alone. Tubes containing 1·0 ml. of lactalbumin medium were inoculated with 3·8 $\log_{10}$ i.u. virus and samples taken at intervals titrated on the chorio-allantoic membrane.

Curve B shows the disappearance and reappearance of infective virus in tissue culture. A number of tissue cultures was infected with 3·8 $\log_{10}$ i.u. and at intervals, tubes were taken, the whole contents ground and titrated on the chorio-allantoic membrane.

Spread in tissue.

In an attempt to ascertain the mode of spread of virus in the tissue, a series of cultures of respiratory epithelium was used.

Firstly, 0·1 ml. of neutralising fowl antiserum was shown to be capable of preventing infection by 10^8 pock-producing doses of ILT strain H.2184 provided that the antiserum was added before the virus. The antiserum alone had no toxic effect.

To each culture 10^4 pock-producing doses of ILT strain H.2184 was added. Eight hours was allowed for adsorption and then 0·1 ml. of antiserum was

added. Two cultures were fixed and stained after 24 hours and two at each succeeding 24 hours up to 72 hours.

When examined it was noted that cultures to which antiserum was added after adsorption of virus, presented the same picture as did cultures to which no antiserum was added. The cytopathogenic effect progressed at the same rate and comparable inclusion bodies were present. It appears that once infection is established the presence of antiserum has no detectable effect on its extension. This suggests that either the virus spreads in the tissue by direct extension from cell to cell, or that sufficient virus is liberated into the fluid to overcome all the antiserum present. The latter is unlikely in that in the infected cultures at no stage could virus be detected in the culture fluid.

As a simple direct test for infective virus in the culture fluid a number of cultures of respiratory epithelium were made on coverglasses and infected with 10^4 pock-producing doses of ILT strain H.2184. After allowing 8 hours for adsorption of the inoculum, the fluid was sucked out, and the culture washed twice using 1 ml. of medium. Antiserum 0·1 ml. was then added and the tubes incubated for 1 hour. The fluid was once more removed and the culture washed three times with 1 ml. of medium each time. These infected coverglass cultures were then placed in the same tube as healthy cultures of the same age, so that the tissues faced away from each other, and incubation continued on the roller drum.

Of twenty-eight healthy cultures placed "back-to-back" with infected ones, six became infected. In four cases the cytopathic changes were evident on the fourth day after being placed with the infected culture and in the other two cases, on the sixth day.

DISCUSSION.

The ILT virus was cytopathogenic for both chick-embryo fibroblasts and chick-embryo respiratory epithelium, complete destruction taking place in 3-6 days and 2-4 days respectively. No tissue reaction of a similar nature to that described by Weller (1953) with varicella vesicle fluid on tissue cultures of human skin-muscle, was observed. Intranuclear inclusions were demonstrated in respiratory epithelium infected with a freshly isolated strain of ILT, but were not seen in infected fibroblast cultures.

It is usual, though not invariable, for virus to appear in the fluid phase of tissue culture. Egg chorio-allantoic membrane infectivity of fluids from infected cultures of various ages was negative in all cases. In an experiment in which infected and uninfected coverglass cultures were grown "back-to-back" a proportion of hitherto normal cultures showed cytopathic changes. This effect could be due either to a little infective virus being liberated occasionally from the infected culture or to the breaking free of infected cells carrying the virus to the uninfected side.

Failure to demonstrate infective virus in fluid from infected cultures may be accounted for by (1) the virus being transmitted directly to adjacent cells without entering the fluid bathing the cells, or (2) virus being liberated intermittently in cycles of multiplication, with insignificant free virus present at times other than at peaks of liberation. Detection would, if this were true, depend on sampling at the appropriate stage of the cycle.

Direct transmission of virus to adjacent cells has been suggested by Wildy (1954) working with the HFE strain of herpes simplex multiplying on the chorioallantoic membrane. Cycles of multiplication are well known in bacteriophage, and have also been shown to occur in influenza virus (Cairns, 1952) and in herpes simplex (Wildy, 1954). Whereas bacteriophage is liberated in a "burst" and readsorbed rapidly, liberation of the animal viruses studied is a steadily increasing "leak", building up to a maximum, and maintaining large amounts of free virus in the fluid for a considerable time, about 4 hours in the case of herpes simplex. It may be that similar events may contribute to the failure to detect ILT virus in the fluid phase of the tissue cultures. Any thermal degradation of virus would also weigh against its detection in the fluids. The fact that cross infection occurs only in a proportion of cultures in the "back-to-back" cultures suggests that the ILT virus spreads in tissue cultures largely by direct cell to cell transmission.

SUMMARY.

The virus of infectious laryngotracheitis of fowls was successfully grown in cultures of chick-embryo tissues. It was recovered from ground cultured tissues, but not from the fluid bathing them.

The virus produced a cytopathogenic effect which was neutralised by specific antiserum.

Intranuclear inclusions were demonstrated in cultures of respiratory epithelial cells infected with a freshly isolated strain of the virus.

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THE BLOOD PRESSURE OF SHEEP[1]

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As a preliminary step in certain experiments it has been found necessary to establish the blood pressure of normal sheep. Previous figures are scanty and only very few animals have been investigated by the different observers. It is therefore thought worthwhile to place on record the present findings.

Du Bois-Reymond (1908) gave the mean blood pressure of the carotid artery as 170 mm. Hg. but does not signify the observer or the number of sheep used. Dresbach (1910) using cocaine, chloroform or ether took readings from the carotid artery (usually the left) by a mercury or a Hürthle manometer and his findings were as follows. Using local anaesthetic (6 animals) the mean blood pressure was 110 mm. Hg., using chloroform (8 animals) 96 mm. Hg. and using ether (5 animals) 103 mm. Hg. He also noted that changes in posture, such as standing, lying on the back or side, could cause a maximum change of 10 mm. Dukes (1955) quotes Dresbach's figures and also states that Dukes and Sampson using local anaesthetic on 13 animals ranging in weight from 27 to 133 lbs. found the mean blood pressure to vary between 90 and 140 mm. Hg., with an average of 114.

Method.

For the purpose of future experiments it was necessary to establish the systolic and diastolic pressures in both the carotid and the femoral arteries. Under intravenous nembutal anaesthesia and with the animal lying on its side, the vessels were exposed through a short skin incision and a 22 gauge inserted into the lumen. The needle was connected to the pressure head of a capacitance manometer by a short piece of polyethylene tubing and the system filled with heparinised citrate solution. The pulse wave was recorded in ink and calibration was performed after each recording against the mercury column of a clinical sphygmomanometer.

Forty animals have been used in all and 52 readings taken. When more than one reading has been taken on an individual animal at least 3 months had elapsed between readings. The age of the animals varied between about three months and three years. They were all hand-fed merino cross-breds consisting of both ewes and wethers.

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RESULTS.

The individual blood pressure readings show a fairly wide scatter (Table 1), but moderately smooth distribution curves have been obtained (Figs. 1-4).

TABLE 1.

The minimum and maximum readings of the various pressures in 52 recordings.

		Minimum mm. Hg.	Maximum mm. Hg.
Carotid Artery	Systolic Pressure	100	180
	Diastolic Pressure	70	141
	Mean Blood Pressure	90	155
	Pulse Pressure	11	48
Femoral Artery	Systolic Pressure	118	191
	Diastolic Pressure	85	142
	Mean Blood Pressure	98	160
	Pulse Pressure	26	57

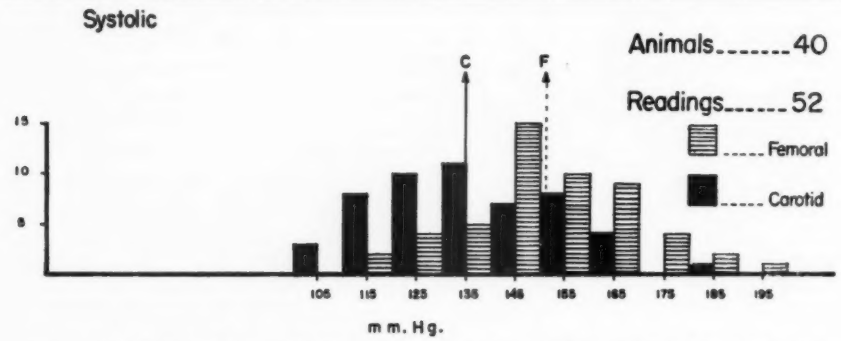

Fig. 1. Histogram of the systolic blood pressure readings in the carotid and femoral arteries. In this and subsequent figures, C. and F. mark the means of the readings of carotid and femoral pressures respectively.

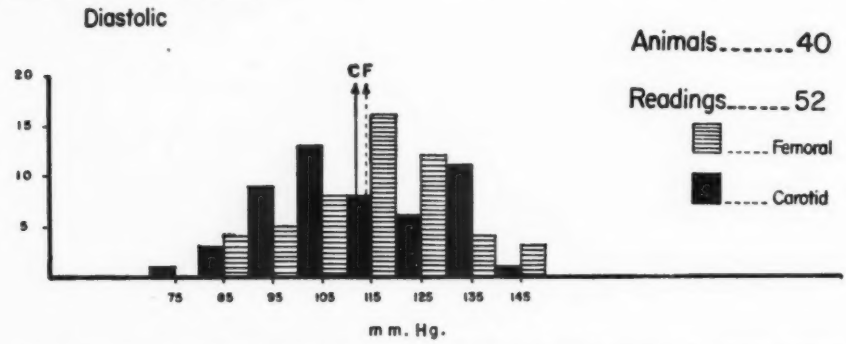

Fig. 2. Histogram of the diastolic blood pressure readings in the carotid and femoral arteries.

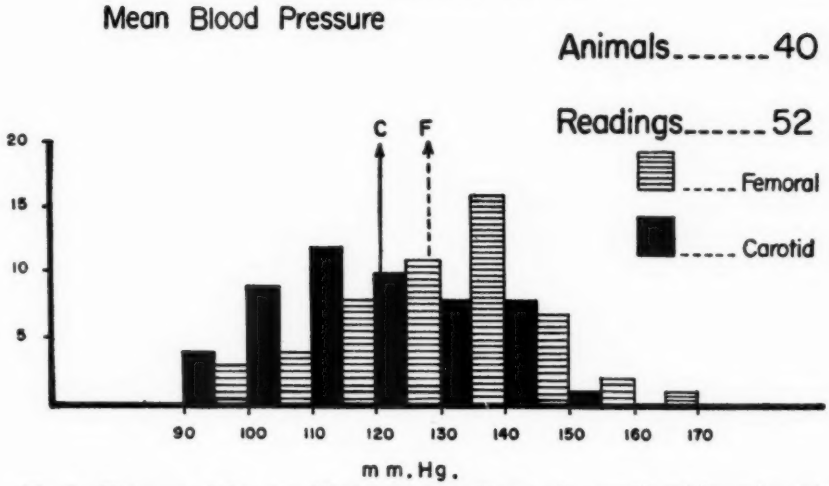

Fig. 3. Histogram of the mean blood pressure readings in the carotid and femoral arteries.

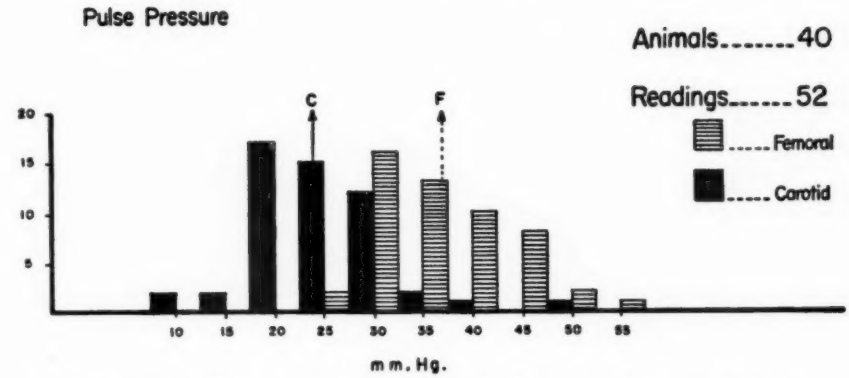

Fig. 4. Histogram of the pulse pressure readings in the carotid and femoral arteries.

TABLE 2.

The various blood pressures calculated from 52 readings.

		Mean mm. Hg.	95 p.c. Confidence Levels	Standard Deviation
Carotid Artery	Systolic Pressure	135·46	130·58 to 140·34	17·95
	Diastolic Pressure	112·0	107·43 to 116·57	16·8
	Mean Blood Pressure	121·61	116·33 to 125·89	15·7
	Pulse Pressure	24·71	22·88 to 26·54	6·74
Femoral Artery	Systolic Pressure	151·04	146·36 to 155·72	17·2
	Diastolic Pressure	114·31	110·23 to 118·39	15·0
	Mean Blood Pressure	127·38	123·18 to 131·58	15·45
	Pulse Pressure	36·54	34·74 to 38·34	6·67

The figures in Table 2 have been derived after the actual readings had been grouped in either fives (Pulse Pressure) or tens (Systolic, Diastolic and Mean Blood Pressures). This accounts for the slight discrepancy between the difference between the systolic and diastolic pressures and the pulse pressures thus calculated.

As an alternative, the figures can be arrived at by the simple process of dividing the sum of the readings by the number of such readings (Table 3) and as can be seen, there is no appreciable difference between the two final solutions.

TABLE 3.

The values for various pressures calculated by dividing the sum of the readings by the number of such readings.

		Average Pressures (mm. Hg.)
Carotid Artery	Systolic Pressure	135·8
	Diastolic Pressure	111·2
	Mean Blood Pressure	121·7
	Pulse Pressure	24·6
Femoral Artery	Systolic Pressure	150·0
	Diastolic Pressure	113·0
	Mean Blood Pressure	126·6
	Pulse Pressure	37·0

An interesting finding is that the femoral systolic and pulse pressures are higher than those in the carotid, the differences being statistically highly significant ($P < 0{\cdot}01$). On the other hand, the differences between the carotid and femoral diastolic and mean pressures are not significant ($P > 0{\cdot}05$).

SUMMARY.

By means of a capacitance manometer 52 intra-arterial blood pressure recordings have been made on 40 normal sheep in both the carotid and femoral arteries.

The mean pressures in the carotid are Systolic 135, Diastolic 112, Mean 122 and Pulse Pressure 25 mm. Hg.

In the femoral the mean pressures were Systolic 151, Diastolic 114, Mean 127 and Pulse Pressure 37.

Acknowledgments. Our thanks are due to the help afforded by the Department of Medical Illustration, University of Sydney, also to Dr. H. O. Lancaster for assistance with the statistics.

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STUDIES ON INTERMEDIARY IRON METABOLISM

XI. THE SOURCE OF IRON IN THE REGENERATED LIVER OF THE RAT AFTER HEPATECTOMY

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(*Accepted for publication 26th March, 1957.*)

The use of the combined operation of partial hepatectomy and splenectomy as a means of removing storage iron rapidly ("operative iron depletion") was reported earlier (Venkatachalam and Kaldor, 1957). In the young male albino rats the liver regained its preoperative weight within nine days of the operation, but its storage iron content was still only one-half of the preoperative value. Twenty days after operation the storage iron in the regenerated liver was found to have risen to the preoperative level. This represents an unexpectedly rapid rate of repletion. Work on dogs and on man (Haskins, Stevens, Finch and Finch, 1952; Fontès and Thivolle, 1936) where serial removal of blood was used to deplete the storage iron showed that iron stores remained deficient many months after blood removal had been discontinued and after the subjects had recovered from the anaemia which had been induced.

This paper describes experiments designed to find the source of iron deposited in the regenerated liver. Two possibilities were considered:

(a) that the loss of storage iron acts as a stimulus for increased absorption of dietary iron; and

(b) that iron liberated from the haemoglobin of destroyed red cells is retained by the regenerating liver at the expense of the circulating haemoglobin mass.

These possibilities were explored in three independent but interrelated experiments. In the first, the previous work (Venkatachalam and Kaldor, 1957) was reproduced with minor modifications. In the second, the gastrointestinal absorption of radioactive iron after operation was measured. In the third, the effects of the operation on the blood volume were studied with the aid of radioactive chromium.

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[1] This work was undertaken during tenure of a Colombo Plan Senior Medical Fellowship. Permanent address: Nutrition Research Institute, Coonoor, India.

[2] This work was undertaken during tenure of a National Health and Medical Research Council Fellowship.

Materials and Methods.

Adult male albino rats were used in all three experiments. Partial hepatectomy and splenectomy were performed in one stage, the former by the method of Higgins and Anderson (1931) with slight modifications. On the day of operation 0·5 ml. of a 1 p.c. solution of neoarsphenamine and 0·5 ml. of a solution containing 10 mg. penicillin sodium salt, and 5 mg. streptomycin calcium chloride were injected subcutaneously. The animals received food and water *ad libitum*. The iron content of the diet was found by analysis to be 20 mg. per 100 gm.

Experiment I.

The aim of this experiment was to determine the amount of storage iron in the liver at varying intervals after operation. Thirty animals weighing between 324 and 443 gm. were used and the removed lobes of the liver and the spleen were weighed. The animals were killed in groups of six at intervals of 4, 8, 12, 16 and 32 days after operation. Six intact rats weighing between 354 and 437 gm. were killed for control measurements. Blood was withdrawn by cardiac puncture and the liver removed and weighed. Haemoglobin, serum iron, latent iron binding capacity and storage iron (non-haemoglobin tissue iron) values were determined by methods described elsewhere (Walsh, Arnold, Lancaster, Coote and Cotter, 1953; Kaldor, 1953; Kaldor, 1954a). The serum protein concentration was measured by the biuret method.

Experiment II.

The object of this experiment was to determine the percentage of iron absorbed from an oral test dose at varying intervals after operation. Four groups of rats weighing between 288 and 445 gm. were used.

Preparation of radioactive iron solution. Radioactive iron in the form of $Fe^{59}Cl_3$ solution was obtained from the Atomic Energy Research Establishment, Harwell, England, through the agency of the Commonwealth X-Ray and Radium Laboratory, Department of Health, Australia. The iron content of the solution was 0·48 mg. per ml. and it had an activity of 87·53 μc. per ml. 2·7 ml. of this solution were made up to 41 ml. with distilled water. To each ml. of this solution, 0·5 mg. iron as $FeCl_3$ was added to make the final iron content of the solution 0·53 mg. per ml. To determine the activity of the test dose an aliquot was set aside for radioassay.

Administration of radioactive iron. The animals were fasted for 24 hours before administration of the test dose. Under light ether anaesthesia, a thin polythene tube was passed into the stomach and 1 ml. of the radioactive iron solution was injected with a syringe, the tube being immediately washed through with 2 ml. of water. The test dose was administered to rats in the four groups at intervals of 1, 4, 10 and 20 days respectively after operation.

The collection of specimens, radioassay of samples and calculation of results were described previously (Brading, George and Walsh, 1956).

Experiment III.

The purpose of this experiment was to determine changes in blood volume at varying intervals after operation. Six groups of rats weighing between 338 and 474 gm. were used.

Cr^{51} labelled red cells. Blood obtained by heart puncture from rats of between 300 and 400 gm. weight was mixed with heparin and centrifuged. The supernatant plasma was discarded. Radioactive chromium solution ($Na_2Cr^{51}O_4$ containing 6 μc. per ml. obtained from the Physics Department, St. Vincent's Hospital, Sydney) was added to packed cells in the ratio of 1 volume solution to 3·5 volumes packed cells. The mixture was incubated at 37° C. for 45 minutes. The cells were then centrifuged and washed three times with physiological saline. A small aliquot of these labelled cells was retained for radioassay, so that the activity of the injected cells could be calculated.

Methods of estimation and calculation. Estimations were made on the different groups of rats at intervals of 1, 3, 10, 15 and 20 days after operation. The femoral vein of the rat was exposed under light ether anaesthesia and 0·5 ml. of labelled cells was injected with a tuberculin syringe. After 10 minutes the chest was opened in the midline and blood taken from the heart into a heparinised syringe.

Haematocrit and haemoglobin values were determined and the volume of the remaining aliquot noted. This aliquot was haemolysed with a small quantity of saponin and assayed for radioactivity, using a scintillation counter. The counter incorporated a thallium activated sodium iodide crystal 5 cm. in diameter, in a lead castle with wall 1-inch thick. The counter impulses were registered on a scaler in the conventional manner. The counter and scaler were of Australian manufacture, supplied by R. B. M. Long Pty., Sydney.

The blood volumes were calculated from the formula:

$$\text{Blood volume (ml.)} = \frac{\text{c.p.m. of labelled cells injected}}{\text{c.p.m. per ml. of rat's blood}}$$

The red cell volume is the product of the blood volume and the haematocrit value divided by 100.

RESULTS.

Experiment I.

The storage iron content of livers and spleens before operation, and the amounts of iron removed at operation, are shown in Table 1. The liver weights were calculated on the basis that the liver is 2·7 p.c. of the body weight, a figure obtained from control rats. Two points require comment:

TABLE 1.

Storage iron content of livers and spleens and amounts of iron removed at operation.

Group	(Mean Values)							
	Body weight (gm.)	Liver weight* (gm.)	Liver iron (mg.)	Spleen weight (gm.)	Spleen iron (mg.)	Liver + spleen iron (mg.)	Liver iron removed (mg.)	Liver + spleen iron removed (mg.)
1	365	9·85	1·22	1·20	0·67	1·89	0·85	1·52
2	398	10·70	1·27	1·10	0·68	1·95	0·93	1·61
3	396	10·70	1·34	1·24	0·83	2·17	0·92	1·75
4	375	10·10	1·19	1·06	0·75	1·94	0·85	1·60
5	375	10·10	1·09	1·35	0·61	1·70	0·79	1·40

* Calculated on the basis of 2·7 p.c. of body weight.

(a) In all groups the iron in the spleen was more than 30 p.c. of the combined total contained in liver and spleen. This is considerably more than was found in young rats used in the earlier work (Venkatachalam and Kaldor, 1957), but it agrees with observations regarding variation with age in the distribution of storage iron in rats (Kaldor and Powell, 1957).

(b) The amount of liver tissue removed at operation was about 70 p.c. of the total liver weight, while only about 50 p.c. was removed in the earlier experiment. This is probably due to improved technique, but it could also be due to a difference in the relative weights of liver lobes in young and adult rats.

TABLE 2.

Storage iron in the residual liver lobes and in the regenerating livers.

Group	(Mean Values)					
	Storage iron in residual lobes (mg.)	Interval between operation and killing (days)	Weight of regenerated liver (gm.)	Liver iron (mg.)	Storage iron added during interval	
					cumulative total (mg.)	per day (mg.)
1	0·37	4	8·40	0·56	0·19	0·048
2	0·34	8	8·98	0·79	0·45	0·056
3	0·42	12	8·15	0·76	0·34	0·028
4	0·34	16	10·03	0·92	0·58	0·036
5	0·30	32	10·92	1·07	0·77	0·024

Table 2 shows the amounts of storage iron in the residual liver lobes immediately after operation and the amounts in the regenerated livers up to the 32nd day. The iron content of the livers, initially low, increased progressively and 32 days after operation was not significantly less than the preoperative value. This is also shown in Fig. 1. The pattern is somewhat different from that found in the earlier work with young rats, and restoration of the preoperative storage iron content is slower. The essential finding is, however, the same, i.e., the liver tissue is fully replenished within weeks of losing more than two-thirds of its storage iron. The last column of Table 2 shows that the amount of iron added per day to the regenerating liver was greatest during the first eight days and

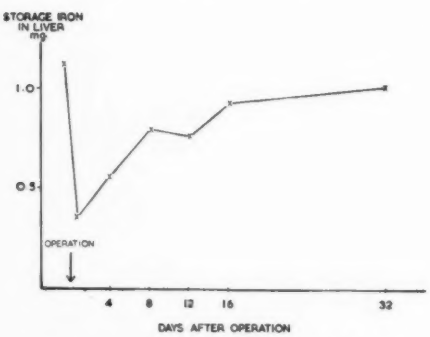

Fig. 1. Storage iron in the livers before and at different stages after operation.

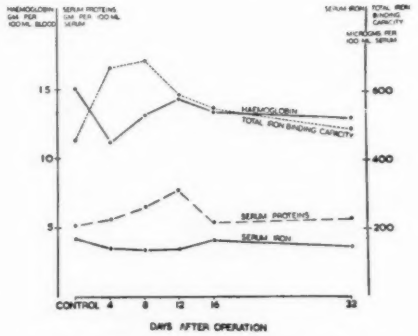

Fig. 2. Haemoglobin, serum proteins, serum iron, and total iron binding capacity of serum.

diminished progressively afterwards. The concentration of haemoglobin was slightly reduced throughout the period of observation, and the concentration of serum proteins rose progressively to the 12th day but returned to the control level by the 32nd day. The serum iron value did not change significantly, but the total iron binding capacity showed the same type of change as the serum proteins. These changes are seen in Fig. 2.

Experiment II.

The amount of iron absorbed and its relationship to the test dose are shown in Table 3. The total iron absorbed into the body was not significantly different in the four groups. The percentage of iron absorbed by rats in all groups was lower than 5·1 p.c., the figure found when using a similar dose in intact rats (Brading, George and Walsh, 1956). The bulk of the absorbed iron was found in the blood, even though the newly regenerated liver was known to be deficient in iron.

TABLE 3.

Absorption of orally administered test dose of iron.

Group	Time interval between operation and test dose (days)	Number of rats	Body weight (gm.)	(Mean Values)					
				Absorbed iron in blood		Absorbed iron in liver		Absorbed iron in blood and liver combined	
				percentage of test dose	mg.	percentage of test dose	mg.	percentage of test dose	mg.
1	1	8	425	3·41	0·018	0·19	0·001	3·60	0·019
2	4	9	391	2·65	0·014	0·20	0·001	2·85	0·015
3	10	9	374	2·31	0·012	0·11	0·001	2·42	0·013
4	20	10	418	2·72	0·015	0·19	0·001	2·91	0·016

Experiment III.

Table 4 gives the results of the blood volume measurements. One day after operation the blood volume was increased but the red cell volume was the same as in the control group; the increase in blood volume was therefore due to an increase in plasma volume. At 3 and 10 days after operation the blood volume was significantly reduced, the red cell volume falling to a greater extent than the plasma volume. Both the red cell and plasma volumes had increased at the 15th day. The increase in the plasma volume was still more marked at the 20th day but the red cell volume was less at 20 days than at 15 days after operation. These changes are also shown in Fig. 3.

TABLE 4.

Mean haemoglobin and blood volume values.

Group	No. of rats	Haemoglobin value per 100 ml blood (gm.)	Body Weight (gm.)	Blood Volume	Red Cell Volume	Plasma Volume
				(ml per 100 gm. body weight)		
Control	6	16·3	402	5·78±0·138*	2·87±0·054*	2·91
1 day after operation	6	15·9	405	6·13±0·218	2·88±0·116	3·25
3 days after operation	4	15·8	359	4·71±0·798	2·18±0·384	2·53
10 days after operation	6	15·4	399	4·08±0·143	1·88±0·100	2·20
15 days after operation	6	15·4	399	5·55±0·118	2·73±0·068	2·82
20 days after operation	5	14·2	366	5·13±0·080	2·21±0·126	2·92

* ±Standard error of mean, obtained from $\frac{\text{S.D.}}{\sqrt{n}}$ where n is the number of observations.

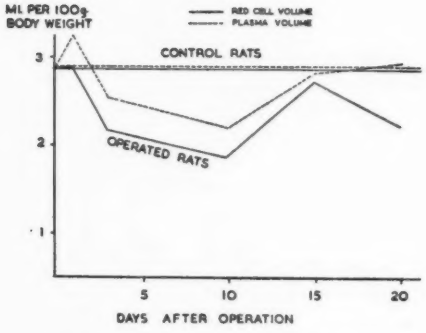

Fig. 3. Red cell and plasma volumes.

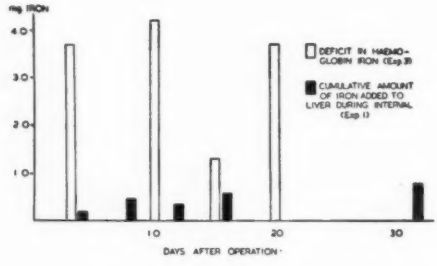

Fig. 4. Figure showing (1) the deficit in haemoglobin iron and (2) the amount of iron added to the liver.

DISCUSSION.

The main object of the three experiments reported in this paper was to determine the source of the iron that was added to the regenerated liver of rats after operative iron depletion. The three experiments yielded the following essential information:

Experiment I. Storage iron in the regenerated liver was restored to the preoperative level in approximately 32 days after operation.

Experiment II. The amount of iron absorbed from an oral test dose at different times throughout a 20-day period following operation did not exceed the amount absorbed in an earlier experiment by intact animals receiving the same dose.

Experiment III. The red cell volume was reduced following operation and the preoperative volume had not been restored by the 20th day.

These results indicate that the iron deposited in the regenerating liver was not obtained by increased absorption of iron from the food but must have been derived from the circulating haemoglobin mass.

Some quantitative aspects of this postulated redistribution of body iron are illustrated by the histogram in Fig. 4. This was constructed from the following two sets of figures:

(a) The cumulative amount of iron added to the liver during different intervals after operation, as found in Experiment I. The figures used in the histogram are identical with those in the second last column of Table 2.

(b) The amounts of iron equivalent to the deficit in haemoglobin mass at different stages after operation. These amounts were calculated from the data obtained in Experiment III as follows:

$$\text{Haemoglobin mass (gm.)} = \text{Total blood volume (ml.)} \times \frac{\text{Haemoglobin (gm.) per 100 ml. blood}}{100}$$

$$\text{Iron equivalent of haemoglobin mass (mg.)} = \text{Haemoglobin mass (gm.)} \times \frac{0{\cdot}334}{100}$$

where 0·334 is Hüfner's figure (1894) for the percentage iron content of haemoglobin.

The iron equivalent of the deficit in haemoglobin mass in a given group of operated rats is the difference between the iron equivalent in control rats and that in the operated rats.

The histogram shows that at all stages up to 20 days after operation the deficit in the iron of the circulating haemoglobin mass was in excess of the amount of iron added to the regenerating liver. The blood volume was not measured beyond 20 days but the trend in the changes observed up to that stage, and the low haemoglobin value on the 32nd day, suggest that the haemoglobin mass was still reduced at that time.

This reduction of the blood volume was probably not due to haemorrhagic loss suffered at the time of operation, because haemorrhage would have been followed in a short time by a compensating increase of the plasma volume. It seems therefore that the reduction in red cell volume liberated a large amount of iron which would be available for the regenerating liver tissue.

The reduction in red cell volume is not easy to explain. A blockage of

marrow function seems unlikely because the rats were able to incorporate absorbed Fe^{59} into haemoglobin at approximately the same rate throughout the 20-day observation period. A haemolytic mechanism could have produced the reduction in red cell volume, but not the associated reduction in plasma volume. It is not clear from the experiments whether removal of the liver tissue or removal of the spleen was responsible for the reduced red cell volume, but it is of interest that one of the authors (Kaldor, 1954b) has found consistently low haemoglobin values in rats after splenectomy. This occurred when the rats were protected against overt Bartonellosis with neoarsphenamine, as in the present work.

Experiment I in this paper differed from work reported earlier only in the age of the rats used. The results are compared below.

	Venkatachalam and Kaldor (1957). Measurements made up to 20 days after operation.	Experiment I (this paper). Measurements made up to 32 days after operation.
Storage iron in regenerated livers.	Little increase during first nine days, then complete restoration.	Gradual increase, but more rapid during first two weeks. Almost complete restoration in 32 days.
Haemoglobin concentration.	Progressive decline.	Decline during first week, afterwards rising to a value slightly below that of the control.
Serum protein concentration.	Progressive decline.	Progressive rise; followed by a fall to normal within 32 days.
Serum iron concentration.	Unaffected.	Unaffected.
Total iron binding capacity of serum.	Unaffected.	Early rise; return to normal within 32 days.

The findings are similar except for the serum protein concentration and the iron binding capacity of the serum. We can offer no reasonable explanation of these differences but assume that they must reflect differences in protein metabolism after hepatectomy in the young and old rats.

SUMMARY.

The operation of partial hepatectomy and splenectomy was used to remove storage iron rapidly. The storage iron content of the regenerating liver was determined at intervals up to 32 days after operation. It was found that the iron content, initially low, increased progressively and its value reached the pre-operative level after 32 days. Experiments were carried out to determine the source of the iron added to the regenerating liver. No increased absorption of

iron was found from orally administered doses of radioactive iron. The red cell volume, determined with radioactive chromium, was reduced after operation and the preoperative value was not restored in 20 days. It was concluded that iron deposited in the regenerating liver was derived from the circulating haemoglobin mass.

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OBSERVATIONS, USING NEW METHODS, OF THE EFFECTS OF NECK COMPRESSION ON WATER-DIURESIS IN RATS

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The Starling views on the physical factors operating at the capillary section of the circulation were adopted for a considerable period (Starling, 1896a, 1896b; Harrison, 1935) to explain the retention of fluid covertly and more especially as frank oedema in congestive cardiac failure (C.C.F.).

An explanation solely in terms of peripheral and cardiac physical factors is no longer tenable and in view of the evidence which suggests that in C.C.F. renal function is affected humorally (e.g. see Blake *et al.*, 1949; Smith, 1951a; "Electrolyte Balance in Cardiac Failure", 1955) it is not surprising that attention has been directed to possible sites, including the neck-head region, involved in a humorally-mediated alteration of renal function.

Experiments involving neck compression in man have yielded results which are varied and not easy of interpretation. Viar *et al.* (1951) have postulated some intracranial "volume sensitive" system. To our knowledge only one author (Fishman, 1953) has reported similar experiments on animals (chloralosed dogs) but with negative results. A particular difficulty in respect to laboratory animals lies in the lack of ready means of inducing cephalic congestion in the unanaesthetised animal.

The present report is concerned with: (a) new methods for the study of effects of neck compression on the urine flow of water-loaded unanaesthetised and anaesthetised rats; (b) the results of such experiments; (c) experiments on a human subject. The results indicate unequivocally an effect with both categories of rats, but not with the human subject.

METHODS.

Experiments on the effects of neck compression on the urine flow of unanaesthetised rats.

Preparation of recovery animals. Males of initial weight 200 gm. to 300 gm. were used from a hooded strain of *R. norvegicus*. The animals were prepared for subsequent

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experiment by an operation in which a sheath of skin was fashioned in the throat to enclose and isolate the trachea and oesophagus from the remaining neck structures, and in such a way that the oesophagus and trachea were not angulated or obstructed during healing or by the application of neck compression. The operations were performed under paraldehyde anaesthesia, 1 ml./kg., given in a split dose in the muscles of each hind quarter. Atropine sulphate, 20 mg./kg., was given subcutaneously 5 minutes prior to the paraldehyde. Both ether and Nembutal were found unsatisfactory in preliminary trials. In the last 40 animals operated under paraldehyde there was a 10 p.c. mortality within the first 24 hours and the bulk of the survivors were satisfactory for experiment.

The details of the operation are as follows. Somewhat X-shaped incisions were made at each side of the neck (see Fig. 1). The skin was freed from the underlying fascia in all directions so that the isthmus of skin was freed centrally, and the external jugular vein was separated from the skin well laterally. The submaxillary glands were isolated and, after clamping the pedicles, were amputated. The stumps were tied. As much as possible of each sternomastoid muscle was excised between artery forceps. The pretracheal muscles were similarly cleared and the greater part amputated. The oesophagus and trachea had now been cleared of all overlying and adjacent muscles and sufficient space had been gained in the neck to permit a skin sheath to be fashioned. The trachea and oesophagus were gently freed from the pre-cervical and carotid sheath from the level of the pharynx to the entrance of the thorax.

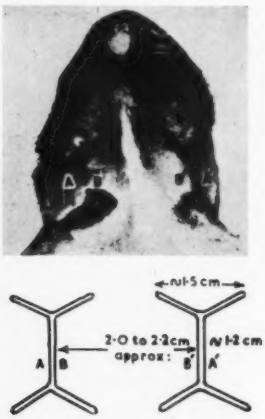

Fig. 1. Neck incision: (A′B′ is an ink mark incision line. AB is an actual gaping incision). Edges A and A′ were sutured together and B and B′.

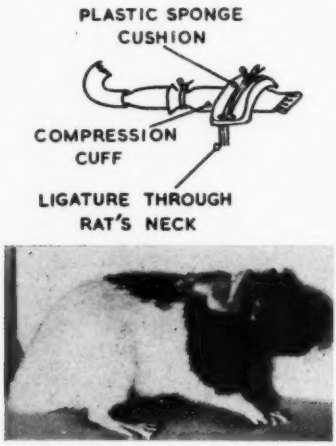

Fig. 2. Compression cuff for the rat's neck and rat with cuff in place.

The skin edges were sutured at their mid-point only, as indicated in Fig. 1. This was found more successful than complete suturing of edges which was more likely to produce dangerous distortion. An aseptic technique was used except that ungloved hands were used for operating. Three thicknesses of coarse thread were passed through the skin-lined tunnel as an obturator. It was imperative to keep this obturator in place at all times except

during experiments since if removed the tunnel would break down and be lost in as little as two days.

The rats all lost weight and condition for a week or more after surgery, but, complications excepted, then proceeded to gain. They were kept at least one month and usually more before experiment.

Experimental technique. The technique adopted appeared to give least discomfort and associated antidiuresis to the rats. The water loading and the application of pressure cuffs (see Fig. 2) to the neck were performed following light anaesthesia in an ether jar. In gavaging, a spring wire stylet in a fine polythene cannula (1 mm. internal) permitted the slight obstruction caused by the skin tunnel to be passed (other methods of gavaging were found not satisfactory). The stylet was withdrawn and the catheter gently advanced into the stomach and then attached to a burette from which the requisite load was measured. The skin tunnel was then threaded with two strands of heavy thread which was tied around the pressure cuff (Fig. 2), firmly in experimental animals and loosely in control animals. The cuff was inflated to 15 cm. of water pressure and tied off. The animals were placed in individual metabolism cages over glass funnels and collecting tubes graduated in one-tenths of a millilitre. The whole procedure took about 5 to 10 minutes to complete after which time the rat was regaining consciousness.

The effectiveness of the means used to elevate cephalic venous pressure was tested in several animals by direct measurement of the pressure in the compressed external jugular vein by means of a column of heparinised saline. The venous pressure was found in each case to be not more than 3 cm. greater than the air pressure in the compressing cuff. On any given experimental day six rats were given 5, 7 and 10 ml. water load with or without 15 cm. H_2O neck compression, the order of experiment being varied in different rats. Continuity of animals was maintained as far as possible. Urine flows were recorded every 15 minutes from 30 minutes after water loading until 150 minutes after loading. Experiments were performed, in a constant temperature room at 22° C., at two-day intervals. Food was withheld for 20 hrs. before experiment, but water was permitted *ad libitum*.

Experiments on the effects of neck compression on the urine flow of rats anaesthetised with ethanol.

Surgical procedure. The preparation used for these experiments was an adaptation of that described by Dicker (1953) for the assay of anti-diuretic activity.

Food was withheld for 16 hours but water was allowed *ad libitum*. The animal was given a dose of 5 p.c. body wt. of 15 p.c. ethanol (B.P.) by gavage. This dosage, greater than that recommended by Dicker, was necessitated by two factors—(i) the strain of animals used showed a reduced anaesthetic response to alcohol compared with albino strains, and (ii) the subsequent neck dissection required a deeper plane of anaesthesia than in assay procedures. Surgical anaesthesia ensued within one hour. The neck and throat were shaved. The bladder was catheterised with 2 mm. bore polythene tubing *via* an abdominal incision which was then closed with wound clips. Neck dissection followed. This consisted of gently separating the relevant structures and inserting a multi-strand ligature dorsal to the oesophagus and trachea, stermomastoid muscles and submaxillary glands and through skin exit holes on either side of the neck. The carotid sheath and main venous channels could thus be subjected to later compression. The oesophagus was incised and the stomach intubated with a filled No. 3 French catheter attached to a burette containing 2 p.c. ethanol. The catheter was tied in place. In this dissection tissue trauma was kept minimal.

Experimental technique. A dose of 5 p.c. body wt. (as a volume) of 2 p.c. ethanol (B.P.) was administered. Urine was collected in narrow measuring cylinders graduated in one-tenths of a millilitre. Volumes were recorded at ten minute intervals. Fluid replacement by stomach tube of 2 p.c. ethanol aliquots was made with every 0·5 ml. increment in

excreted urine. The criterion for a satisfactory preparation was a steady urine flow of 0·9 ml. or greater per minute. This could only be realised if the operating time was reduced to about 20 minutes.

Two types of control were used. At first a replica of the means of neck compression was applied to the thigh. This did not lead to any effect on renal function at the pressures used so it was discontinued. It was possible that the neck compression-renal function effect might be due to general stimulus of pain receptors. To eliminate this possibility a second control method was used in which the internal ligatures were sawed continuously back and forward (subsequently called "sawing"). It was considered that this would provide a greater stimulation of pain endings than would continuous pressure.

Experiments on the Human.

Constant water loading. In these experiments an effort was made to study urine flow in the unanaesthetised adult human under conditions of constant water load with and without neck compression. The same subject (G.W.) was used throughout. The subject took no food for 12 hours prior to experiments but fluids were taken *ad libitum*. No coffee or tobacco was allowed on the morning of the experiment. The urine was voided voluntarily and the initial loading commenced at 0930 hours with four doses of 500 ml. of tap water at 20 minute intervals. Thenceforward voiding was at 15 minute intervals. Immediately after the first post-load micturition, water was taken equal to the volume of urine (v_1) voided. Five minutes later a further volume of one-third of (v_1) was taken and at ten minutes a further volume of one-third (v_1) was taken. Immediately after voiding the second volume (v_2), a volume of water equal to (v_2) minus 2/3 (v_1) was taken. At the 5 and 10 minute periods, volumes equal to 1/3 (v_2) were taken. After the third urine volume (v_3) was collected, a volume of water (v_3) minus 2/3 (v_2) was taken. This procedure was maintained for the duration of the experiment. Neck compression was effected *via* two connected standard sphygmomanometer cuffs. The subject remained seated during experiments except when voiding and drinking.

Single load. The pre-experimental conditions were the same as above. In these experiments the urine flow was studied following the administration of a single water load of 2 litres, which was taken immediately after voiding, in 500 ml. doses at 5 minute intervals. After the fourth dose the bladder was again emptied. This was repeated at 10 minute intervals.

A series of control experiments was performed without neck compression to establish renal volume response to these conditions.

Statistical method.

The urine flows (see Results) were analysed by the standard method of analysis of variance.

RESULTS.

Unanaesthetised rats. The urine let-down was recorded at regular fifteen minute intervals over a period of one hundred and fifty minutes, yielding flow curves for this period. As it is not possible readily to analyse such curves *in toto*, two parameters were selected which would be likely to reveal an antidiuresis if this were present. These were (i) the time taken for an animal to let down 10 p.c. of its water load, and (ii) the total urine volume after 150 minutes.

A typical set of results is shown in graphical form in Fig. 3.

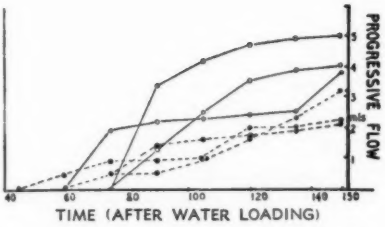

Fig. 3. Urine let-down in a typical experiment on water diuresis in unanaesthetised rats. Solid lines ———— no compression. Broken Lines — — — — compression. Each set of points is derived from consecutive urine volumes (as progressive totals) from one animal of the six used on this day. The water dosages to each animal are omitted from the figure, since when these results are taken with all others, variation due to dosage was found to be not significant.

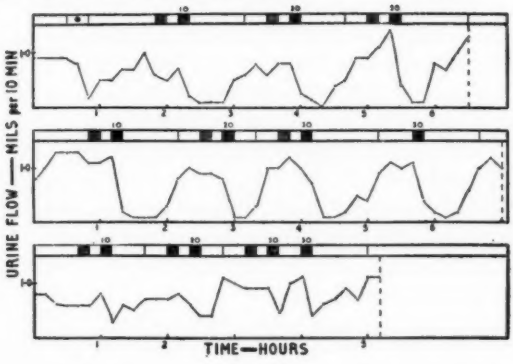

Fig. 4. Experiments on water diuresis in ethanol anaesthetised rats.

||| represents a period of control "sawing" (see text).
■ represents a period of compression with the pressure in cms. of water.
* this flow depression was due to acute respiratory distress due to mucus.

The antidiuretic effect of neck compression in the water loaded rat was by both measures, highly significant ($P < 0{\cdot}01$).

In simple water loading of intact rats *per os*, diuresis would be virtually complete after 3 hours. The total volume of urine from a rat at this time would be expected to be significantly different for doses of 5, 7 and 10 mls. In our experiments such a difference was not found. This could have been due to the relatively small numbers of animals used in view of the high variability associated with such things as preceding anaesthetic and manipulation and voluntary micturition. This did not obscure, however, differences in diuresis due to neck compression.

Anaesthetized rats. In a series of eight preliminary experiments involving a variety of applied pressures and times of application, a reduction in urine flow always followed neck compression at applied pressures between 7 cm. and 30 cm. of water. In three of these experiments in which two different pressure levels were applied for 10 minutes each, there was a greater and more extended diminution of flow following the higher pressure. There was in no experiment a significant diminution of flow following control pressure (on the leg) or control "sawing".

The results of these experiments made possible an experimental design by means of which could be determined (a) the qualitative effect of neck com-

pression on urine production and (b) the magnitude of this response with respect to different applied pressures.

In these final experiments of a standardised design, pressure was applied at 10, 20 and 30 cm. water pressure with each compression period preceded by a control period of neck "sawing". Suitable periods of observation were interpolated between treatments to permit detection of effects on urine flow and also to permit urine flow to return to the steady basal condition.

The results of these final experiments are presented in graphical form in Fig. 4. There is no apparent difference in response to the different applied pressures in these three experiments so they were not continued, as adequate evidence of the qualitative effect of compression had accrued. The flow following compression was reduced in four instances to 50 p.c. or lower and in seven additional instances to 10 p.c. or lower of the mean pre-compression flow.

Human. In the human experiments with matched output-input there was a progressive reduction in diuresis on all days. It was, however, very variable in extent even though the same subject was used throughout. Within the limits of the trials performed, neck compression did not appear to influence the pattern of diuresis.

Again in the experiments where urine flow was measured following one large dose of water, although a high degree of day to day reproducibility was obtained, neck compression did not alter the pattern of diuresis, as compared with controls.

DISCUSSION.

The results from the experiments with both rat preparations indicate clearly that the neck compression caused an anti-diuresis after water-loading. Although, as is obvious from the experimental design, the results in conscious animals do not lend themselves to any close analysis of the time-course of events relating to neck compression, the ethanol-anaesthetised preparation does indicate within a matter of minutes what is occurring. Fig. 4 shows that the anti-diuretic response to neck compression is apparent within five minutes and that on release of compression the response carries on for the order of thirty minutes before diuresis reaches its former level. These responses are of importance not only in relation to a discussion of the nature of the mediation of the response, but also because they constitute a sensitive indicator in determining the first broad link in the chain of events. There are several aspects of the compression technique each of which must be considered as a possible initiator of the anti-diuretic response. We are convinced that the potent feature is the venous congestion in the neck-head region, because the following alternatives appear to be reasonably excluded on the grounds submitted:

(a) *Non-specific tissue compression.* The control experiments with pressure applied to the thigh, and impinging for part of the circumference on bare subcutaneous tissue, did not produce any alteration to the urine flow. In addition,

the degree of pressure applied is not great enough to lead one to expect that tissues, other than those specifically receptive, would be affected. The oesophagus and trachea were deliberately excluded from pressure because these are known to be sensitive structures.

(b) *Pain and discomfort.* These are known to induce anti-diuresis. The intact skin of the recovery animal would appear to protect the rat from actual pain while control animals carried the thread and the uninflated cuff on their necks so that they had neck discomfort, albeit no pressure was applied. More cogent is the fact that in the ethanol-anaesthetised rats, the neck ligature was "jiggled" in such a way that it is almost certain that more pain and discomfort would be produced than with pressure alone. Nevertheless, as Fig. 4 shows, no anti-diuresis resulted.

(c) *Stimulation of the carotid sinus region.* The application of the ligature and compression were well below the sinus and there does not seem any likelihood of direct stimulation. Nor does it seem likely that the pressures used could directly affect the carotid blood pressure or supply. It is, of course, not possible, from our experiments, to exclude the possibility that the region was stimulated in some way by venous congestion.

(d) *Interference to the respiratory passages.* In both types of experiment the technique by-passed any direct pressure on the oesophagus and trachea. It might be considered that the skin tension after compression could produce some obstruction or irritation. However, in most animals there was no visual evidence of such an action; the control tying did not exhibit an effect in the unanaesthetised rats, and some animals had obviously adequate looseness of the skin to prevent pressure.

(e) *Dislocation of haemodynamics.* Any considerable alteration of venous return could be expected to affect cardiac output, etc., and renal blood flow. We felt that, with the pressures used, there would be only a temporary hold-up with cephalic venous return, quickly followed by relatively normal flow in dynamic equilibrium.

We consider that the anti-diuresis was initiated by venous congestion cephalic to the compression site, but we have no indication as to the determinant vascular area, e.g. jugular, intracranial or intraspinal veins.

The absence of a similar response in the human, previously demonstrated by a number of authors, e.g. Lewis (1950), Viar *et al.* (1951), Lombardo and Harrison (1953), is here confirmed by us. Again Fishman (1953) has reported this response absent in the chloralosed dog. The rat response is therefore of great interest in relation to the former observations and the question arises as to whether the discrepancy is due to (1) a phylogenetic difference in this function between rat and man and dog, or (2) the possibility that the response exists in man and dog but that it may be obscured by other factors with which the experiments to date have not been designed to cope.

A phylogenetic difference could only be ruled out by careful experiment since other differences in renal function between rat and man are already known, e.g. see Smith (1951b), Dicker (1956), Eggleton (1956).

In our view alternative (2) above, demands serious consideration. Several factors could have been operating during reported observations to "swamp" or hide the antidiuretic response in humans: (a) the internal jugular vein lying in the angle between the relatively rigid trachea and sternomastoid muscle may be protected from some or all of the low pressure applied to the neck. (It is to be noted that this cannot be the explanation in Fishman's (1953) work with the dog as he directly measured the pressure in the internal jugular vein.) (b) There are, in man, pronounced diurnal variations in urine flow (e.g. Sirota *et al.*, 1950) which were evident in our experiments also and which may mask a slight but significant antidiuretic response. (c) Posture alone affects both sodium and water diuresis (e.g. Brun *et al.*, 1945; Lewis *et al.*, 1950, and Viar *et al.*, 1951). Moreover, in the human, the antinatriuresis of the upright posture can be reversed by the application of compression to the neck. It is not clear whether such compression has also an effect on water diuresis; since, by comparison with the reduced urine flow in the upright position, the flow during neck compression appears to be at the higher rate encountered in the recumbent position. Such an effect, if substantial, would be opposite to that which we have observed in the rat.

We have not tested the effect on the rat of prolonged neck compression but presumably it would lead to water retention, at some point a new water equilibrium being reached. As long as such an effect as we have observed in the rat cannot be ruled out in man and other animals, the possibility remains that a cephalic venous congestion-water relationship may be potent in the genesis and maintenance of the complex syndrome of congestive cardiac failure.

Although our experiments allow no decision as to whether the observed response in the rat is neural or humoral, the duration of the antidiuresis after cessation of neck compression suggests that either there is a long-lasting after-discharge of the nervous system, or more likely a humoral mediation is involved. In the latter case a neuro-hypophyseal-renal effect or an anterior-pituitary adrenocortical effect appear as likely possibilities. In order to clarify some of the speculative issues our observations have raised, studies on other relevant renal functions are needed.

SUMMARY.

New methods are presented for the study of the effects of neck compression on the water diuresis of rats, unanaesthetised or anaesthetised with ethanol.

The results, in both preparations, indicate that venous congestion in the cephalic area induces an antidiuresis in water loaded animals.

Neck compression experiments on an unanaesthetised human male showed no such effect and were in confirmation of earlier findings.

The possible significance of the observations to the syndrome of congestive cardiac failure is discussed.

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TISSUE METABOLISM DURING POSTERIOR REGENERATION IN THE EARTHWORM

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Earthworms have been extensively used for regeneration studies. Many have contributed to the morphogenetic aspects of the problem, from Reaumur and Bonnet in 1742 to Moment and Gates in 1950 and 1951. Segmentation and its reconstitution has provided a convenient and accurate measure of regeneration. The capacity for complete regeneration varies with the segment level at which transection occurs. Anterior regeneration on a posterior substrate, that is the reconstitution of a head end differs from the converse that is posterior regeneration on an anterior substrate. The differences are those of regeneration rate, of the degree to which the number of segments removed is replaced and of the extent of the zone in which a normally functioning regenerate is produced.

This paper is concerned with tissue metabolism during posterior regeneration at the 60/61 segment level and forms a part of an investigation of the relationship between the metabolism of the regenerate, the axial metabolic gradient and the capacity for complete reconstitution. Preliminary work has shown that the post-embryonic growth curve tends to flatten out after eight weeks. The results described herein refer to worms at least fifteen weeks old, thus eliminating variations in regeneration rate which occur prior to and during the logarithmic phase of growth, that is up to the eighth week. The relationship between the number of new segments, the length of the regenerate and regeneration time was determined so that an estimate could be made of variation between worms. Sampling an experimental batch was then based upon regenerate length and segment number at any given time since the degree of morphogenesis, not the period of regeneration, is the interest with reference to metabolism.

By ten days a small non-segmented bud or blastema, 1·0 to 1·5 mm. long, has developed. At six weeks the regenerate has attained full segment number (35 to 40), a length of 12 mm. and is growing rapidly. After ten weeks, rapid growth in length has given way to a slow overall increase approximately the same as the normal increase of this age group, whereas segment diameter and pigmentation are still subnormal. The length at ten weeks is about 25 mm.

Tissue was investigated from three zones (see Fig. 1). Zone 2 is a five-segment zone in the substrate adjacent to the regenerate R. Zone 1 is the ten-

segment region immediately anterior to zone 2. Zone 1 in a ten-day regenerate appears histologically normal. In zone 2 there are mitoses throughout the three to four segments adjacent to the blastema. Most cell divisions appear in, or associated with, the epithelium of the alimentary canal and in those cells forming or adhering to the intersegmental septa. In the regenerate itself mitotic activity is evident at the approximate interface between the normal and the regenerating tissue. At six weeks mitoses are distributed fairly evenly throughout zones 2 and R1 but are much fewer than at the ten-day stage. In the ten-week regenerate only an occasional mitosis can be seen.

Material and Methods.

The earthworm *Eisenia foetida* (Sav.) was cultured and tissue preparations obtained as previously described (O'Brien, 1948). The worms were sexually mature and clitellate, of approximately the same size, being taken from a culture fifteen to twenty weeks old. There appears to be no significant difference between clitellate and non-clitellate specimens in rate or degree of regeneration at the 60/61 segment level. In many but not in all cases the clitellum had disappeared by the tenth week of regeneration. Specimens were transected at the 60/61 intersegmental furrow and kept in batches of 100 to 500 while regenerating. Normal worms were also set aside and starved to provide control estimations at ten days and six weeks. The regenerating worms do not ingest food until about the third week at this level of injury. Regenerating cultures were sampled at ten days, six weeks and ten weeks. The regenerate (zone R), the adjacent zone of five segments (zone 2) and the ten-segment zone anterior to this (zone 1) were taken for analysis. Normal feeding worms and uninjured, starved worms were used for tissue between segments 55 and 65 to provide controls. The aspects of metabolism investigated were: glycogen content, glycolytic activity, endogenous oxygen consumption and succinoxidase activity. The methods used are referred to in an earlier communication (O'Brien, 1957).

EXPERIMENTAL RESULTS.

Glycogen.

A considerable amount of glycogen is present in both the body wall and viscera of large worms. The glycogen content of the entire worm increased to a value between 6·5 and 7·0 mg./gm. wet weight at about six weeks after hatching. The body wall glycogen reaches a maximum at approximately four weeks, while the visceral glycogen continues to increase. During six weeks starvation the glycogen content decreased by less than 50 p.c. from 5·0 to 3·0 mg./gm., the rate of decrease being greatest during the first ten days.

Glycogen was estimated in the regenerate and in two adjacent segment zones. Three lots of 200 regenerating worms were sampled at ten days, six weeks and ten weeks after injury. Thirty worms were selected at each sampling from each lot. The selection was not random but based on equivalence in length of regenerate and number of new segments. In this way a fairly wide individual variation was reduced, and this procedure was followed throughout. Fig. 1 describes the results so obtained.

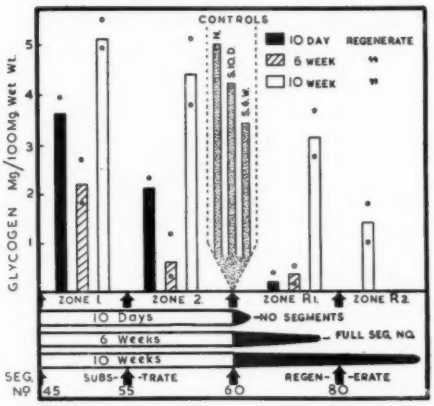

Fig. 1. Glycogen content of regenerating and adjacent tissue. Control estimations at segment level 60 in normal feeding (N) and in starved worms (S.10.D and S.6.W.) are represented by the stippled histograms at level 60. Each histogram is the average of several estimations, the maximum and minimum values being represented by the small circles associated with each histogram. Glycogen is expressed as mg. per 100 mg. wet weight of 'whole' worm.

At the blastema stage of regeneration the glycogen content of the regenerate is very low (0·2 mg./gm.). The five-segment zone of normal tissue adjacent to the blastema shows a glycogen depletion from 3·6 to 2·1 mg./gm. while the next ten-segment zone indicates a value lower than the normal (ten day, starved control) at that segment level. It would appear that during the stages of blastema formation, glycogen reserves are metabolised more rapidly than they are replaced. At six weeks when full segment number has been attained the glycogen of both regenerate and adjacent tissue is still very low, whereas at ten weeks it is normal in all but the regenerate where it has greatly increased.

Glycolysis.

Both lactic and pyruvic acids are normal constituents of earthworm tissue. Aerobic values for whole worms expressed as micrograms of acid per 100 mg. live weight were lactic acid 10·5, pyruvic 1·7. Following a period in nitrogen, lactic acid increased to 63·5 while pyruvic acid fell to 1·0. Manometric estimations of total acid when compared with estimated lactic and pyruvic indicate that they are the only acids present in significant amounts. With regard to regeneration, the glycolytic activity of the blastema (ten days) was about 80 p.c. above the normal value for that level (ten days, starved). The five normal segments of zone 2 showed a rise of some 68 p.c., while in zone 1 normal values prevailed. At six weeks the glycolytic activity of regenerate and adjacent segments had dropped but was still considerably greater than normal. At ten weeks the regenerate retained a higher glycolytic rate than level 60/61 but had almost returned to a value that might be expected from its altered position with reference to the gradient curve (O'Brien, 1957). This suggests that glycolytic mechanisms may be the predominant ones during the formation of the blastema and its subsequent early differentiation.

TABLE 1.

Anaerobic glycolysis of regenerate.

Minced Tissue

Controls: (1) Normal feeding 119
(2) Starved 10 days 128
(3) Starved 6 weeks 113

Regenerate age	Zone 1	Zone 2	Zone R_1	Zone R_2
10 days	111	190	223	—
6 weeks	119	145	173	—
10 weeks	116	118	143	160

Homogenised Tissue

10 days	45	64	88	—
6 weeks	44	58	70	—
10 weeks	42	50	60	68

Controls: (1) Normal feeding 21
(2) Starved 10 days 21
(3) Starved 6 weeks 20

Results expressed as micrograms of lactic acid per 100 mg. wet weight of tissue mince or its equivalent in the homogenate, per 60 min. 2 p.c. glucose used as substrate in a glycolytic reaction system essentially similar to that used for vertebrate muscle. Temp. 27° C.

Endogenous oxygen consumption.

The entire sample was minced. No effort was made to examine different tissue preparations at this stage. Samples of the regenerating zone taken earlier than ten days included more substrate than regenerate, the cut being made at the second last intersegmental furrow. At this early stage the injured infolded end was filled with a relatively acellular semi-fluid plug. By ten days the blastema had formed but some of the original tissue was unavoidably included. From fourteen days the sample is entirely new tissue.

The results, Fig. 2, curve A, show low QO_2 values up to four days at which time no significant quantity of new tissue had formed. The drop in respiratory activity is possibly magnified by there being less active tissue per mg. of sample than at later stages where differentiation and growth have begun. The experiment was repeated and the respiratory activity obtained as μl. of O_2 per mg. total nitrogen. These values were then expressed as μl. of O_2 per 100 mg. wet weight, deriving the latter values from analyses of normal whole worm. The results substantiate the drop in QO_2 following injury but suggest that the extent of the fall is less than the first experiment would indicate. Fig. 2, curves B and C, indicate this comparison. B represents QO_2 as μl. of O_2 per mg. total nitrogen, while C represents the B-values calculated as μl. of O_2 per 100 mg. wet weight.

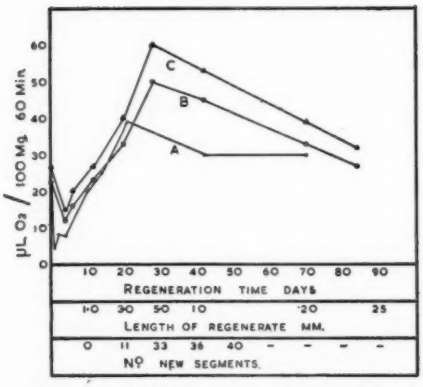

Fig. 2. Endogenous oxygen uptake by minced regenerate, at 27° C. and pH of 7·3. Curve A is a direct representation of results obtained as µl. of O_2 absorbed per 100 mg. wet weight of mince per hour. Curve B represents oxygen uptake obtained directly as µl. of O_2 per mg. total nitrogen per hour, and Curve C the same results calculated as µl. of O_2 per 100 mg. wet weight per hour. The amount of tissue per flask was the same in all cases. The regeneration time in days is related to the length of the regenerate in mm. and to the number of new segments. From thirty to fifty worms used for each estimation up to six weeks after which fifteen were taken.

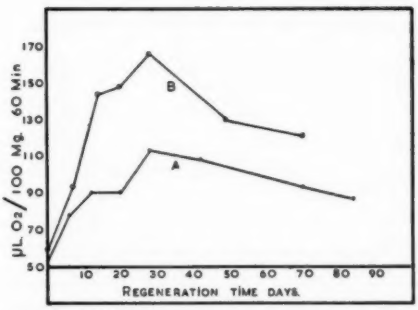

Fig. 3. Succinoxidase activity during regeneration. Curve A represents minced tissue, Curve B homogenised tissue. Results expressed as µl. of O_2 absorbed per 100 mg. wet weight per hour. Temp. 27° C., pH 7·5.

From ten days after injury samples of 'new' tissue can be obtained and analyses of wet weight, dry weight and total nitrogen showed that there was no significant difference between regenerate and substrate at the segment level concerned. These results are expressed in Table 2 and indicate that the values for the endogenous respiratory activity of the mince are not greatly influenced by variation in water content of the tissues or variation in the total nitrogen.

TABLE 2.

Wet weight, dry weight and total nitrogen.

Seg. level 60 Regen. time	Wet Wt. mg.	Dry Wt. mg.	Total N. mg.	Dry Wt./ Wet Wt.	Dry Wt./ Total N.
0	100	17·7	1·21	0·177	14·6
12 days	100	16·4	1·13	0·164	14·5
6 weeks	100	17·5	1·25	0·175	14·0
10 weeks	100	17·4	1·23	0·174	14·1

Regeneration time 0 refers to tissue taken from normal worms at level 60, the alimentary canal full of soil and detritus being removed before weighing. The twelve day regenerate contains slight amounts of 'old tissue' whereas the six and ten week regenerates are new tissues entirely.

The oxygen uptake increased to a maximum value at three to four weeks then declined at six weeks to a value which remained higher than the normal range for segment level 60. By ten to twelve weeks there was little difference between the QO_2 of substrate and regenerate at level 60.

Succinoxidase activity of regenerate.

Minced and homogenised tissues were assayed for succinoxidase activity over much the same period of regeneration as in the previous experiment. The result approximately parallels that of endogenous activity (Fig. 3, curve A minced tissue, curve B homogenate). There is a definite increase reaching a maximum at approximately twenty-eight days, followed by a decline. There does not appear to be any initial drop in the succinoxidase activity comparable with the endogenous respiration. An effort was made to obtain values at daily intervals for six days following transection. The results were so variable that no definite pattern emerged. It is felt that the difficulty of obtaining a sample of early blastema 'uncontaminated' with 'old tissue' is probably the major cause. In no case, however, was a significant decline noticed during this period.

DISCUSSION OF RESULTS.

Estimations of glycogen content have been frequently used to yield information about the type of metabolism prevailing at different stages of development. Much of the information about the metabolism of fertilisation in the eggs of invertebrates stems from glycogen changes. The distribution of glycogen during gastrulation (Heatley and Lindahl, 1937) has demonstrated the existence of two gradients in the blastula and that intense utilisation of this glycogen accompanies invagination of the gastrula which results in a glycogen-rich epidermis and neural plate, and a glycogen-poor endoderm and mesoderm. Similar results were demonstrated in the toad and the frog by Tanaka (1934) and in *Discoglossus* by Brachet (1935). The importance of glycogen as a metabolic substrate in early growth and differentiation is well summarised by Needham (1942) who points out that data from amphibian, avian and mammalian embryos conform to this pattern.

The utilisation and reformation of glycogen during regeneration in *E. foetida* conform to the findings for other organisms; an early stage of glycogen depletion corresponds to the early development and subsequent differentiation of the blastema and this is followed, on completion of differentiation, by a gradual increase of glycogen during a period which is characterised by the absence of segment differentiation and a falling growth rate.

Preliminary work indicated that a system of anaerobic glycolysis is present in the worm tissue which corresponds in its essentials with that of mammalian tissue; the question whether lactic acid production is a valid estimate of glycolysis among worms is important as many invertebrates are known to produce

other acid products. Most of the earlier work relating to worms is indicative of a glycolysis producing a mixture of acids; however, Davis and Slater (1928) found and identified lactic acid in earthworm tissue and were of the opinion that it was the only acid formed. In this work with *E. foetida* both lactic and pyruvic acids were found as normal aerobic tissue constituents.

Although other data are unavailable for earthworms, similar results have been obtained in numerous differentiating and growing systems. The avian egg shows a high peak in lactic acid at five days corresponding to early developmental stages. Early segmentation in the frog is uninhibited by anaerobic conditions, but the capacity for further development becomes more and more dependent on aerobic respiration. Generally speaking, work on amphibian egg development has demonstrated an abrupt metabolic change at gastrulation when a great increase occurs in rates of glycogenolysis and glycolysis. Gastrulation heralds the onset of structural differentiation and might be considered to correspond to the situation which exists when the blastema of the regenerating worm begins to differentiate (approximately five to ten days after injury). The regenerating amphibian limb and tail show the high lactic acid values associated with the tissue of the blastema, and the subsequent decline with the onset of differentiation and growth.

The aerobic side of carbohydrate metabolism in earthworm tissue follows in all essentials the cytochrome linked cycle of enzymes common to almost all aerobic systems yet investigated, among the metazoa. In regeneration, aerobic respiration showed a decline following injury, at which time the glycolytic cycle is apparently in active operation. As the capacity for anaerobic glycolysis declines, the aerobic metabolism as represented by the endogenous oxygen uptake and by assayed succinoxidase activity increases to a maximum by the fourth to sixth week; the peak in aerobic metabolism followed soon after full segmentation. As the regenerate approached morphological normality, this declines to a steady value in excess of the normal value for level 60/61, but consistent with that to be expected from the gradient curve.

At this segment level the onset of rapid growth in the regenerate precedes the differentiation of full segment number and it appears that the peak in succinoxidase activity approximates in time the onset of rapid growth and is related to growth rather than to differentiation. This observation is supported when regeneration is initiated at segment level 40/41 and a level ten segments from the posterior end. At 40/41 the differentiation of full segment number precedes the onset of rapid growth and the maximum succinoxidase activity; in the tail region full segment number, maximum succinoxidase activity and the onset of rapid growth occur at the same time.

SUMMARY.

During the ten days following transection at segment level 60, the regeneration blastema is formed. From ten days to six weeks segment differentiation

and growth occur. From six weeks to ten weeks there is no further segmentation, growth alone taking place.

While the blastema is forming there is a decrease in glycogen, more particularly in the region of the injury. This continues until segmentation is complete, after which the glycogen concentration increases once more.

Glycolytic activity is greatest during blastema formation and remains high until segmentation is complete. By ten weeks the activity is normal.

Endogenous respiration at first shows a drop following injury then a rapid rise to a maximum at about four weeks. Values gradually return to normal by ten weeks. The peak in respiration is considered to be related to the onset of a period of rapid growth in the regenerate.

Succinoxidase activity shows no evidence of an initial drop but continues to rise to a maximum at about four weeks, after which it declines gradually to a normal value at ten weeks.

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PAIN-PRODUCING SUBSTANCES FROM THE STINGING BUSH *LAPORTEA MOROIDES*

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The severity and long duration of the sting of the *Laportea* spp. (Urticaceae) have been known and publicised in eastern Australia since the middle of the 19th century. As early as 1860 Bennett described how horses which had been stung had to be rested for several days before they could be used again. In 1887 Bailey and Gordon reported that "the stinging hairs have frequently caused the death of horses". Bancroft (1889) observed that "after being nettled, one is reminded of the fact for several days, and in exceptional cases for weeks whenever the nettled part is wet". The three species of these nettles found in eastern Australia, two of which are trees and the third a shrub, occur mainly in rain forests. In the past these were a danger to railway construction gangs. Nowadays, timber workers and bushmen are the main sufferers. The Gympie bush is regarded as a menace to stock and particularly to horses which, if severely stung, become violent and have had to be shot. Men, too, become frantic if badly stung and Francis (1955) has reported seeing a man unconscious some time after falling onto a stinging shrub. There is no record of the death of any human victims in Australia (Webb, 1948), but Winkler (1922) reported a human fatality in New Guinea following the sting of *L. condata* which, however, is not found in Australia. On the other hand, mild stings from nettles have been used by Aboriginals to treat rheumatism (Bailey, 1909), and furthermore, the fruits borne by the Australian species are edible.

The sting occurs when the skin is pierced by the rigid hairs found on the leaves and green stems of these nettles. Many theories of the mechanism of the sting of these and other stinging plants have been advanced. Petrie (1906) claimed that free formic and acetic acids were present in the fluid contained in the hollow siliceous hairs of *Laportea* spp. and he considered that the amount of these acids in a large hair was quite sufficient to cause the severe sting. Pammel (1911) stated that it seemed that the urticaria resulting from nettle stings was caused by a plant toxin. The U.S. Dispensatory 21st ed. (1926) reported that an alkaloid, a non-volatile albuminoid and a glucoside were found in *Urtica dioica*. Flury (1927) concluded that the sting of *Urtica dioica* was produced

by a non-volatile, unsaturated, nitrogen free compound of acid nature with properties resembling the resin acids and that the nettle poison was neither formic acid, nor an enzyme nor a toxalbumin. Emmelin and Feldberg (1947) found acetylcholine (ACh), histamine and an unidentified smooth muscle-contracting substance in the hair fluid of *Urtica urens* after they had set out to investigate the suggestion of Haberlandt (1886) that the active poison was an enzyme. Broadbent (1953) concluded that the trichomes of cowhage (*Mucuna pruriens*) which produce itching, contained a histamine liberator. Later, Bowden, Brown and Batty (1954) found that cowhage contained 5-hydroxytryptamine (HT) whilst Arthur and Shelley (1955) have claimed that the active principle in cowhage is the endopeptidase, mucunain. Recently, Collier and Chesher (1956) produced evidence of the presence of HT in *Urtica dioica*.

Rechter (1949) and Shaw and co-workers (1955) observed that extracts of *Laportea* leaves behaved as mixtures of ACh and histamine, with respect to their actions on preparations of smooth muscle and on the cat's blood pressure. The only other work performed on the pharmacology of the stinging material from *Laportea* spp. appears to be that of Bancroft (1889) and of Francis (1955). The former described the effect of contact with stinging hairs and noted that the commonly accepted antidote, *Alocasia macrorrhiza*, the cunjevoi, was of no value. He observed that the juice of the leaf was tasteless, that it gave no reaction to litmus and that when injected into frogs it had no apparent effect. Francis has described the gross effects of the sting and has noted the effects of heating and of drying the leaf on the activity of the stinging material therein. He made the interesting observation that a herbarium specimen, which had been stored for 46 years, was still active.

With this background an attempt has been made to investigate some pharmacological aspects of the *Laportea* stinging material more fully. Because Francis (1955) reported that of the three *Laportea* species, *L. moroides* gave the most vicious sting in both intensity and duration, that species was used in the experiments described here.

METHODS.

Since pain appeared to be the primary effect of *L. moroides* action, the essential test was the reaction of human skin to stinging hairs or extracts of the hairs. Other pharmacological actions were sought on a variety of tissues and preparations.

Extracts of stinging material. Several thousand hairs from the leaves and green stems of *L. moroides* were removed with fine forceps, transferred to 0·9 p.c. NaCl solution and ground in a mortar. During the preparation of these extracts it was noticed that anyone plucking the hairs was subject to bouts of sneezing. The saline extract was passed through a sintered glass filter, the filtrate varying from a clear to a slightly opaque liquid sometimes with a greenish tinge. These filtrates were used in the early experiments, but later were subjected to dialysis before being tested. Dialysis was effected by suspending a cellophane bag, containing the extract, for 3-5 hours in distilled water which was frequently changed. The procedure was deemed to be complete when the dialysate failed to cause an immediate and swift contraction of the isolated guinea pig intestine.

The cellophane membranes used were found to be freely permeable to trypan blue (MW 960), but they retained a solution of inulin (MW 5000) without loss, for 3 days.

Both undialysed or dialysed extracts were stored at 0° C.

Cutaneous reaction. Extracts of stinging hairs (in 0·9 p.c. NaCl) were injected intradermally in volumes of 0·02-0·04 ml. The forearm skin of 6 subjects was used in these tests.

Frog and toad rectus abdominis. The preparations were suspended in a 6 ml. bath in Locke solution, diluted with distilled water in the ratio of 1 : 1·4, containing eserine 10^{-5} and aerated with oxygen.

Isolated intestine. Rabbits and guinea pigs were killed by a blow on the back of the neck. A 4-5 cm. portion of the upper ileum was suspended, after emptying its lumen, in a 10 ml. organ bath in Mg++-free Tyrode's solution containing either 0·10 p.c. or 0·11 p.c. $NaHCO_3$ and oxygenated with 100 p.c. O_2 or 95 p.c. O_2 and 5 p.c. CO_2 respectively. Contractions of only the longitudinal muscle were recorded with a frontal writing lever which had a fivefold magnification.

Segments of ascending colon were obtained from rats anaesthetised with ethyl chloride and then bled to death by cutting their throats. The colon was set up in the same manner as the ileum, but was suspended in modified Locke solution containing one-eighth the usual amount of calcium.

Isolated uterus. Young adult virgin female rats were used, some of which had received stilboestrol (100 μg./kg. intramuscularly) in oil on the previous day. One horn of the uterus was suspended in a 10 ml. bath in a modified Locke solution (Gaddum, Peart and Vogt, 1949). The recording lever had a tension of 1 gm. and a tenfold magnification.

Blood pressure. Cats were anaesthetised with sodium pentobarbitone (30-40 mg./kg. I.V.). Blood pressure was recorded from the carotid artery and respiration *via* a tracheal cannula and a metal bellows recorder (Palmer). Drugs were administered *via* the femoral vein.

Hind limb perfusion. Rats were killed by a blow on the back of the neck. The abdominal cavity was opened and after the rectum and the inferior and superior mesenteric arteries were divided between ligatures and the intestines were removed, the abdominal aorta was cannulated and the body wall and vertebral column were cut through above the point of cannulation. Locke's solution was passed through the cannula and the flow of venous effluent was recorded using a drop counter. For toads, the Locke's solution was diluted 1 : 1·4.

Drugs: All drug doses given in the text refer to the salts. These were nicotine acid tartrate, atropine sulphate, histamine acid phosphate, mepyramine maleate, acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, tryptamine hydrochloride, adrenaline hydrochloride and dibenamine hydrochloride. The enzymes were commercial samples.

RESULTS.

Cutaneous reactions to nettle leaf. The results of contact with the stinging hairs of the *Laportea* spp. have been broadly described by Webb (1948) and by Francis (1955). The first sensation following penetration of the skin by the rigid hairs is a slight itch. This is followed in less than 60 seconds (usually 15-20 seconds) by a severe prickling effect which quickly becomes an intense pain. This pain sensation is complex. It is composed of a background of tingling on which is superimposed an intermittent stabbing pain, with sharp radiations passing in all directions. After a time, which can be as short as 10 minutes with severe stings, the stabs of sharper pain decrease in intensity and in frequency,

whilst the background increases to give diffuse pain. The sharp tingling is increased by touch or cold.

Referred pain in the thorax, chin, forehead and in the opposite limb may also be experienced within 5 minutes of the sting. Bancroft (1889) observed that "if the hand be the part nettled the secondary pain starts in the spot nettled and runs up the arm and down the corresponding side." Frequently pain is felt also in the axilla and this may persist for several days longer than the referred pain already described. The duller background of pain may last for several days and even when it finally disappears the affected portion may be hypersensitive for 2 months and become extremely painful if rubbed, or exposed to cold, but not to warm water. This area may also become spontaneously painful in cold weather, particularly in the morning.

Simultaneous with the production of pain is the initiation of an effect comparable with the triple response to histamine. The nettled area quickly becomes covered with 1 mm. diameter red spots which fuse to form a zone of swelling and erythema. The surrounding area over a diameter of 4 cm. from a 1 cm. focus, frequently develops discrete axon reflex vasodilatations, which soon merge to form a flare. This appears within 5-10 minutes and persists for 8 hours or more. Piloerection and sweating also occur. Sweating develops after 5 minutes and is confined to the central zone of erythema. Piloerection is intermittent and sometimes is still present after 10 hours. Sweating, however, is continuous and reaches its maximum after 6-8 hours. Some sweating persists up to 30 hours.

When hairs were gently inserted into the skin using forceps no pain was felt during periods of up to 10 minutes; but if the exposed hairs shafts were rocked with forceps, pain was invariably experienced within 30 seconds. Presumably this movement causes the hair tips to break and release the fluid contained within the hairs. Francis (1955) and others have noted that on microscopical examination a line across the hair near the tip can be seen and it is here that fractures most readily occur.

Intradermal injection of extract. Better controlled dose-response patterns were obtained by injecting a filtered saline extract of crushed hairs intradermally. With this technique the shortest latency for the initiation of the sharp stabs of pain was 10 seconds and that for piloerection was 30 seconds. Vasodilatation was observed within 2 minutes of the injection and sweating within 5 minutes.

These responses have appeared with both normal hair extracts and with those dialysed for up to 3 days — the latter containing no significant amounts of ACh, histamine or HT-like substances. The addition of ACh, histamine and HT, to the dialysed material in quantities sufficient to produce final concentrations similar to those in the normal extract, did not appear to alter the responses which the latter alone elicited.

Rubbing the extract into unbroken skin produced none of the responses associated with its injection. Dialysed extracts of whole leaves produced re-

sponses similar to those of the hairs alone on injection. No obvious tachyphylaxis occurred with intradermal injection in man.

Synthetic pain-producing mixtures. Emmelin and Feldberg (1947) reported that injections of ACh and histamine mixtures produced burning pain. In the present experiments when mixtures containing up to 0·1 mg. ACh and 0·1 mg. histamine in 0·02 ml. were injected, only a transient dull pain was experienced for 5 minutes. It had none of the piercing quality of the *Laportea* extract nor was it as intense or prolonged as the effect of 0·02 ml. dialysed hair extract. Yet 0·1 ml. of the dialysed extract failed to cause an immediate contraction of isolated guinea-pig ileum in a 10 ml. bath, which indicated an ACh and histamine content of less than 0·02 μg. of each in 0·02 ml. Such amounts of these two drugs when injected intradermally failed to produce any responses. Petrie (1906) found 0·002 p.c. formic acid and 0·177 p.c. acetic acid in the nettle and thought that such concentrations were sufficient to produce the sting, but a mixture containing similar amounts of these acids elicited no responses on intradermal injection in the present work.

Blockade of cutaneous reaction. Prior injection of the skin with 0·02 ml. 2 p.c. lidocaine hydrochloride delayed the onset of the responses to the injection of the extract by 10-15 minutes. The reactions were compared with control injections of dialysed hairs. No pain, piloerection or axon reflex flare was observed during this period (in 3 trials), but as soon as the local anaesthesia had worn off the responses were indistinguishable from those of the control injection site. The admixture of the extract with atropine and mepyramine, to give final concentrations of each of 3×10^{-3}, delayed the initiation of pain and piloerection for 5 minutes, during which time the weal area was insensitive to pin pricking. Within 15 minutes, however, the pain responses of the test were similar to those of the control injection. Previous injection of 0·02 ml. tryptamine 5×10^{-3} also delayed the appearance of responses to the extract injected into the same site. But this dose of tryptamine had a local anaesthetic effect as it prevented the perception of pin pricking as well as the axon reflex causing piloerection when ACh 10^{-4} was injected 4 minutes later. The normal response to ACh had recovered when tested at 60 minutes. In all three cases, i.e. with lidocaine, atropine, mepyramine and tryptamine, within 2 hours the effect of the antagonist had entirely vanished while the pain sequence of the *Laportea* extract continued. Consequently these drugs had no effect on the late pain skin reaction and hypersensitivity of the area.

Reputed antagonists. There are many local legends regarding cures for the *Laportea* sting. A number of these suggested antidotes was tested. The method used was to induce similar stings on each arm of 4 subjects either by applying active leaf (1 cm.^{2}) or by injection. One area was treated with the test substance, the other with a placebo. The subjects were not informed of the treatments given and in some trials they were blindfold. The recommended

therapies include rubbing the juice exuded from the leaves, stem and root of *Alocasia macrorrhiza* (cunjevoi) into the affected part, treating with the bark of *Laportea gigas* and the root of *Bowenia spectabilis*, a saturated aqueous solution of picric acid, a saturated solution of sodium carbonate, a solution of ammonium hydroxide, adrenaline cream and mepyramine cream. Some temporary relief was obtained whilst the "remedy" was being applied, but a similar alleviation was produced by rubbing in tap water. Within 2 minutes in all cases the pain became apparent again and no difference between the test and control stings could be distinguished then or on the hypersensitive area which developed later. Rubbing was the essential component of the temporary relief obtained in all the treatments. The only substance tested which on occasion appeared to have some permanent effect was the ammonium hydroxide solution. This was not conclusive, however, as this partial relief occurred on only two of the four occasions on which it was tested.

Frog and toad rectus abdominis. Preparations in eserinised Locke solution contracted when doses (each equivalent to 100 hairs) of undialysed extract were added to the bath. These responses were of the same order of magnitude, of latency and of duration as those to ACh at a concentration of 2×10^{-7}. When dialysed extract equivalent to 100 hairs was added no response was obtained nor was there any alteration to the effect of ACh added simultaneously.

Guinea pig ileum. Doses of undialysed extract equivalent to 1-5 hairs produced sharp contractions with very short latencies in this preparation. Incubation with human blood at 37° C. for 10 minutes or heating to 80° C. for 15 minutes with 0·5 N NaOH greatly reduced the potency of the extract. Hexamethonium 2×10^{-5} which blocked the effects of equiactive doses of nicotine (10^{-6}) did not affect those of the extract. Atropine 5×10^{-8} and mepyramine 3×10^{-9} each reduced the responses to the extract while both antagonists, when administered simultaneously, completely blocked this quick response even to a dose equivalent to 20 hairs. By using these antagonists separately in the above specific doses it was possible to determine the ACh-like and histamine-like activities of these extracts. In five experiments, it was found that the content of each hair was equivalent to 0·01-0·025 μg. ACh and 0·025-0·05 μg. histamine.

When a dose of dialysed extracts equivalent to 20-100 hairs was added to the bath, a different type of response was obtained compared with that produced by the undialysed material. Slower contractions, which took about 30 seconds to reach their maximum height, were preceded by longer latencies of the order of 30 seconds. This contrasted with the 5-8 second latencies following less effective doses of ACh and histamine. Hexamethonium 5×10^{-5}, atropine 5×10^{-8} and mepyramine 2×10^{-8} did not reduce these responses to the dialysed material although they effectively blocked the responses to nicotine, ACh and histamine respectively. It was not possible to make an extensive pharmacological analysis of the active non-dialysable substance because of the

tachyphylaxis which was encountered in every experiment in which it was used. Sometimes only one response to the dialysed material was obtained in an experiment although intervals of up to 60 minutes between doses were allowed during which the muscle behaved normally with respect to other stimulants. The responses to ACh and histamine were not at all affected by previous administration of the dialysed extract.

Rabbit ileum. Doses of undialysed extract produced sharp contractions with short latencies. Doses of dialysed extract equivalent to 500 hairs produced slow contractions after latencies of approximately 60 seconds. This effect was not abolished by hexamethonium 5×10^{-4}. The marked self-blocking action of the active material on this preparation also prevented further analysis by using other drug antagonists.

Rat colon. Here specific doses of atropine greatly reduced the effect due to the undialysed extract. There was a residual effect, however, in the presence of atropine 2×10^{-6} which was reversibly blocked, as were matching responses to HT (5×10^{-9}) after the preparation had been exposed to tryptamine 2×10^{-4} for 10-20 minutes. It was calculated that each hair contained approximately 0·001 μg. of HT-like activity.

Isolated rat uterus. In the presence of atropine 5×10^{-7} responses to undialysed extract were blocked as were those to HT, by prior contact of the muscle with tryptamine 5×10^{-4} for 10 minutes or with dibenamine 5×10^{-6} for 10 minutes. It was estimated on this preparation also that one hair contained approximately 0·001 μg. HT-like activity. Dialysed extracts elicited contractions in this preparation, but again tachyphylaxis did not permit any qualitative work to be done.

Blood pressure in cat. In four experiments only inconsistent results were obtained when blood pressure records were made after intravenous administration of extracts. In the first cat which had been given atropine 1 mg./kg. and mepyramine 1 mg./kg. I.V. the injection of the equivalent of 175-500 hairs of undialysed extract produced an immediate but short and small fall in blood pressure. Four minutes after the administration of the extract there was a substantial pressor response which lasted for about 8 minutes. Yet in another cat which also had been treated with atropine and mepyramine the equivalent of 700 hairs of the same extract when injected had no pressor effect but only a transient and slight depressor effect.

In the other two cats, doses of dialysed extract equivalent to 400-2000 hairs produced no marked effect on respiration and again there was only a transient depression of blood pressure response. The latter was unaffected by hexamethonium 1·5 mg./kg., but was absent after the animals were given atropine 1 mg./kg. I.V. Incubation of the extract with whole blood at 37° C. for 30 minutes destroyed the activity.

It is possible that the transient depressor effects mentioned were due to

ACh in the extract for doses equivalent to 175-500 hairs of undialysed extract would contain 2-10 μg. ACh-like activity which may have a slight action in an atropinised animal. In dialysed extracts possibly not all the ACh had been removed and in the case of the dose equivalent to 2000 hairs an ACh residual of only 1 p.c. would be expected to have an effect.

Hind limb perfusion. In toad and rat limbs, in which adrenaline 10^{-7} in the perfusing fluid was effective in causing vasoconstriction and 50 μg. ACh injected into the fluid effected vasodilatation, undialysed extracts equivalent to 300 hairs produced no response.

Rabbit and guinea-pig eye. Instillation of 0·1 ml. dialysed extract equivalent to 120 hairs into rabbits' and guinea pigs' eyes produced no effect on the pupillary diameter and no irritation or inflammation within 24 hours.

Physical and chemical properties of the pain-producing substance. There was no discernible loss of the activity of the pain-producing substance when hair extracts were dialysed against glass distilled water for 4-6 hours. Even after 72 hours' dialysis against running tap water, considerable pain-producing material was retained by the cellophane membrane. This membrane was readily permeable to trypan blue (MW 960) but was impermeable to inulin (MW 5000). Both undialysed and dialysed extracts equivalent to 2000 hairs/ml. in glass distilled water had a pH of 5·8-6·0, similar to that of the water.

Filtered, dialysed extracts in distilled water or in 0·9 p.c. NaCl were initially colourless, but within a few days when stored at room temperature or at 0° C. they became green and later on brown in colour and showed a flocculent precipitate. The solution filtered free from the precipitate was slightly less effective in producing pain than the mixture containing the precipitate. Boiling the dialysed extract for 10 minutes did not significantly reduce pain production. Warming the extract in 5 N HCl to 65° C. for 5 minutes produced no apparent loss of activity but boiling in 5 N HCl for 1 hour caused almost complete destruction. Boiling in 0·25 N NaOH for 5 minutes caused only 50 p.c. loss of activity. Hair extracts in distilled water, in 0·05 N HCl and in 0·05 N NaOH, were extracted with anaesthetic ether which was then evaporated off and the residue taken up in saline. All three resultant solutions evoked the typical pain response when injected intradermally. Extractions of the same dialysed solution, however, in distilled water with five equal volumes of ether left approximately 50 p.c. of the original activity in the aqueous phase.

Dialysed extracts containing the equivalent of 600 hairs/ml. showed no abnormal U.V. absorption spectrum, but only a fairly uniform decrease in optical density with wavelength when tested on the Unicam spectrophotometer between 210 and 310 mμ.

Electrophoresis for 30 minutes at 3 microamp./cm. in a barbitone buffer pH 8·4 ($\mu = 0{\cdot}05$) caused no migration of the active material from the point of origin on Whatman No. 1 paper.

In an ion exchange column, 10·5 cm. long and 4 ml. in volume, containing Amberlite IRC 50 in equilibrium with 0·1 M NaH_2PO_4 the active material (equivalent to 1000 hairs) in 2 ml. 0·1 M NaH_2PO_4 was recovered in the first 6 ml. of eluant collected at the rate of 4 ml./hour. ACh and histamine (30 μg. and 60 μg. respectively) under these conditions were not recovered before twice that volume had been collected and then the former well before the latter.

The undialysed and dialysed material containing the equivalent of 2000 hairs/ml. caused no digestion of exposed X-ray film gelatine when left in contact at 37° C. for 5 hours, in contrast to the actions of solutions of proteolytic enzymes.

When the dialysed material was incubated with trypsin, pepsin, emulsin and with papain in final concentrations of 10^{-3} at 37° C. for 22 hours and then boiled for 4 minutes, there was no reduction in the pain produced on intradermal injection compared with control solutions. When diastase, takadiastase and hyaluronidase in final concentrations of 10^{-3} were incubated with dialysed extracts at 37° C. for 22 hours and then boiled for 4 minutes, slight but definite reductions in the activities of the solutions were observed.

Ethanol was added to the extract to a final concentration of 90 p.c. and left for 3½ hours at room temperature. Although no precipitate was visible the solution was centrifuged and the upper portion of liquid withdrawn and evaporated at 60° C. under reduced pressure. The evaporating vessel was then washed out with 0·9 p.c. NaCl and the washings, when injected intradermally, were found to contain very little pain-producing activity compared with control samples.

When the extract was left for 2 hours at room temperature in 10 p.c. trichloracetic acid and then filtered (although no precipitate was visible) the resultant solution after neutralisation had not lost a significant amount of activity compared with the controls on intradermal injection.

Boiling dialysed extract with conc. H_2SO_4 and conc. HNO_3 for 10-20 minutes produced no colouration or evolution of gas. Picric and phosphotungstic acids when added to the extract gave no precipitates.

Boiling dialysed extract with conc. H_2SO_4 and then testing for the presence of a reducing sugar with Somogyi's reagent did not give a positive result.

DISCUSSION.

Emmelin and Feldberg (1947) investigated the active substances in the hair fluid of *Urtica urens*. They found 0·01-0·4 μg. ACh and 0·005-0·22 μg. histamine in each stinging hair together with a third unidentified smooth muscle contracting substance. Collier and Chesher (1956) explored the possibility that this third substance may have been HT and indeed, in the sting of *Urtica dioica*, they found this substance to the extent of approximately 0·004 μg./hair. In the present work on *Laportea moroides*, 0·01-0·025 μg. ACh-like, 0·025-0·05 μg. histamine-like and 0·001 μg. HT-like activities per stinging hair have been found. Since the values for ACh and HT-like activities correspond on two

different test tissue preparations, most probably they actually represented ACh and HT. Yet the three substances mentioned above are not predominantly responsible for the painful sting for, if these in concentrations corresponding to the values quoted are injected together intradermally in human subjects, they produce very little effect compared with extracts of *Laportea* hairs. It was difficult also to distinguish between the effects of undialysed extracts and those of extracts from which ACh, histamine and HT had been lost by dialysis. There must, therefore, be at least one other extremely active substance in *Laportea* hairs. This may not be present in *Urtica* spp. for Emmelin and Feldberg did not ascribe much importance to the non-ACh and non-histamine fraction of their extract and Collier and Chesher found no evidence of the existence of a fourth substance in their nettle sting.

In the present work very little of the pharmacology of the unidentified substance was elucidated because of its lack of effect on isolated preparations and because of the small inconsistent effects on the blood pressure or respiration of the cat. It was identified consistently only by its production of pain in human subjects. The material was observed, however, to cause a slow contraction of isolated smooth muscle after a long latency. The response was not entirely reproducible because of the marked self-blocking actions exhibited by the substance. It was observed also that in the guinea pig ileum, hexamethonium, mepyramine and atropine in doses which specifically blocked the actions of nicotine, histamine and ACh did not block the effect of this unidentified material in the dialysed extract. Schachter and Thain (1954) have reported that in large doses the addition of bee vemon to guinea pig ileum in the presence of mepyramine causes a delayed slow contraction which is rapidly desensitized. No actions were noticed on perfused blood vessels in rats' and toads' hind limbs, on the frog's rectus abdominis or when instilled into mammalian eyes.

The mechanisms of the effects of the dialysed material when injected intradermally into human skin have not been fully analysed, but some points may be considered. The pain sensation occurred after 15-30 second latency. The latency suggests that either the active substance is produced on contact with tissues, or that it takes some time to diffuse to pain-nerve endings. Injected material must be in contact with pain nerves since the injection itself is immediately painful. The large size of the active molecules (indicated by dialysis behaviour) could delay diffusion into the nerves, but the constancy of the latency in any one subject suggests that an activation takes place in the skin.

There was very little histamine-like itch. The protracted dull pain, with tingling on cooling or brushing the skin, seems to involve a considerable change in pain-nerve function, which may last four weeks. Analysis of these changes has yet to be undertaken.

Axon reflexes account for the arteriolar vasodilatation patchily distributed over 20-30 cm^2., and the piloerection appears to spread over axon reflex paths.

The central erythema involves direct action on capillaries and arterioles, and the occurrence of sweating only in this area suggests that sweat glands were directly stimulated. Failure of antihistamines to prevent erythema, sweating and flare makes it likely that other substances than histamine are released by the stinging materials. The pain, vasodilatation, sweating and piloerection are very similar to the events produced by Cubomedusae (sea wasp) nematocysts.

Those physical and chemical properties of the pain-producing substance examined do not give much indication of its chemical nature. It is likely to have a molecular weight of 1000 or more because of its retention by the cellophane membrane used. It caused no digestion of gelatine and it was stable to boiling, so that it was obviously not a proteolytic enzyme like that found in cowhage (Arthur and Shelley, 1955) or indeed any kind of enzyme. That the material was not protein-like or polypeptide-like in nature could be inferred from the following: (a) the pH of its solution was similar to that of the water in which it was dissolved; (b) it was not precipitated by picric, phosphotungstic or 10 p.c. trichloracetic acids; after the addition of trichloracetic acid to the active solution it was observed that all the activity remained in solution even had there been a precipitate too slight to notice, and (c) no loss of activity occurred on incubation with pepsin, trypsin or papain. On the other hand, there was a considerable loss of activity when the active aqueous solution was treated with ethanol to give a final concentration of 90 p.c.

The possibility of the substance being an alkaloid was dismissed by the lack of colouration and of evolution of a gas when boiled with concentrated H_2SO_4 or concentrated HNO_3 and its failure to precipitate with picric or phosphotungstic acids.

As incubation with emulsin did not reduce the activity and as boiling with conc. H_2SO_4 did not cause the liberation of a reducing sugar the substance did not appear to be a glycoside.

Although active solutions on standing became discoloured, the active material dissolved in water did not change the pH and there was no peak in the U.V. absorption spectrum to indicate the presence of a double bond. The substance is therefore unlikely to be a resin acid.

Diastase, takadiastase and hyaluronidase reduced the activity of the pain-producing substance by a slight but significant amount when incubated at 37° C. for 22 hours. This allows the possibility of the substance being polysaccharide-like and this field is considerably narrowed by the fact that boiling with concentrated H_2SO_4 did not cause the liberation of a reducing sugar.

It is possible that some of the negative results obtained in the chemical tests were due to insufficient concentrations of the pain-producing substance in the extracts. Possibly a larger scale extraction procedure should be developed before any further chemical testing is undertaken. Here it should be mentioned that Flury's (1927) method of separating *Urtica* spp. hairs is not success-

ful when applied to those of *L. moroides* (Shaw, 1955). Besides the dialysis technique of isolating the pain-producing substance from ACh, histamine and HT the ion exchange resins may prove useful, for in the present work using Amberlite IRC 50, some separation was effected. The active substance is very stable and retains its pain-producing powers for over 40 years in the dry state. Further work on the chemistry of this material should reveal some interesting and powerful substance.

SUMMARY.

Extracts of the stinging hairs of the Gympie bush (*Laportea moroides*) have been found to contain 0·01-0·025 μg. acetylcholine-like activity, 0·025-0·05 μg. histamine-like, and 0·001 μg. 5-hydroxytryptamine-like activity per hair.

Intradermal injection of dialysed extract, free of acetylcholine, histamine and 5-hydroxytryptamine, causes the characteristic intense pain, flare, piloerection and local sweating of a *Laportea* sting.

Specific blockade of the action of acetylcholine, histamine and 5-hydroxytryptamine did not prevent the production of nettle pain.

A stable, non-dialysable substance, resistant to heat, neutral in reaction, unattacked by proteolytic enzymes appears to be the essential pain-producing material. It also induces sweating, piloerection and arteriolar dilatation.

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